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RELATION OF FEEDING IN THE SEA URCHIN STRONGYLOCENTROTUS DROEBACHIENSIS TO DIVISION IN SOME OF ITS ENDOCOMMENSAL CILIATES[1]

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Seven species of ciliates, which are generally regarded as commensals, have been reported from the digestive tract of *Strongylocentrotus droebachiensis* in the Mt. Desert Island region (Powers, 1933a; Beers, 1948). Four of them (*Cyclidium stercoris, Plagiopyla minuta, Euplotes balteatus,* and *Trichodina* sp.) are of erratic occurrence and are excluded from the present study, which is therefore based on the remaining three, namely, the holotrichs *Entodiscus borealis* (Hentschel), *Madsenia indomita* (Madsen), and *Biggaria gracilis* (Powers). They are of almost invariable occurrence in any urchin whose test exceeds 10 mm. in diameter, and the number of individuals of any one of them in an urchin 25 mm. in diameter or larger may be enormous. They were present in all the urchins of the present study.

Although the food of the three has not been studied critically, the evidence indicates that it is largely bacterial. In some gastrioles of *E. borealis,* Powers (1933b, p. 129) reported "rod-like bodies which resemble bacteria," although in others he noted objects which look like the nuclei of intestinal epithelium cells. In *B. gracilis* he reported "bacteria and bits of algae" (1933a, p. 112), but the nature of the food of *M. indomita* was unmentioned. I have observed rod-like structures and short filaments, which were undoubtedly bacteria, in the gastrioles of all three ciliates, but some unidentified material was also present.

Various aspects of the autecology of the ciliates have been treated elsewhere (Beers, 1948, 1961). Findings pertinent to the present study and reported in 1948 may be summarized as follows. *Biggaria gracilis* is essentially an inhabitant of the rectum, which, except in drastically starved urchins, always contains food remnants. In the usual adequately fed urchin *E. borealis* occurs primarily in the stomach and *M. indomita* in the intestine. The regions of the gut being ill-defined, the distribution of the ciliates is not a rigid one. If an urchin is kept without food for a week or longer, the stomach and intestine gradually become empty and the distribution of *E. borealis* and *M. indomita* changes, in that they shift toward the rectum, where they mingle with *B. gracilis.* Dividing specimens of *B. gracilis* can be found in

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[1] Aided by a grant from the Research Council of the University of North Carolina.

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practically any urchin that is collected in nature, but it is a remarkable fact that dividing specimens of *E. borealis* and *M. indomita* are extremely difficult to find, even in ciliate populations of great density. Of 182 urchins that contained immense numbers of both ciliates, only six had dividing specimens of *E. borealis* and only three those of *M. indomita.* (Powers, 1933b, likewise had difficulty in finding dividing forms of *E. borealis* and indeed found only three in all his material, which was evidently plentiful.) It was concluded that division occurs cyclically in the two ciliates: that "short periods of intense divisional activity . . . alternate with long periods of non-divisional life" (p. 111). Since the urchins appeared to be adequately fed and to contain ample bacteria to support the ciliates, the conclusion implies that the cycles are inherent in the ciliates.

I was never entirely satisfied with the conclusion, for cycles of the kind postulated are generally absent in ciliates. When interruptions of division occur, they are usually associated with environmental inadequacies or with special physiological states, such as senescence, autogamy, conjugation, or the production of resting cysts. Since the feeding habits of the urchins were uncontrolled in my earlier study, I returned to the subject in the summer of 1962, in an effort to ascertain whether the division of the ciliates is in any way related to the food of the host. More precisely, the study concerns the following questions, the first two of which are somewhat preliminary, whereas the third is the principal one. (1) What is the condition of the ciliates with respect to division in urchins that have been deprived of food for a considerable period, meaning about two weeks? Earlier observations (unpublished) indicated that dividing forms of *E. borealis* and *M. indomita* are absent in such urchins, but the condition of *B. gracilis* was unrecorded in my notes. (2) Hence, are dividing forms of *B. gracilis* also absent? It is logical to expect a cessation of division in the absence of food, and this expectation was readily confirmed. (3) Then, if these starved urchins are again supplied with ample food, is division resumed in *B. gracilis* and is a cycle of division initiated in *E. borealis* and *M. indomita*?

Materials and Methods

From time to time during the summer, specimens of *S. droebachiensis* were collected at low tide from the rocks and shallow water near the Mt. Desert Island Laboratory. In size they varied from 25–70 mm. in diameter (test, excluding spines); most of them measured 40–55 mm. across. Injured specimens (spines damaged or integument abraded) were rejected, for these may be attacked and eaten by healthy ones. The urchins were transferred in groups of 60–70 to aquaria through which sea water flowed constantly. In these, they were kept without food for two weeks or longer (usually 14–16 days). Voided fecal pellets, which may be re-ingested, were removed daily by siphoning.

Several representative urchins (usually 5 to 10) of each group were opened and examined as soon as collected, and the condition of their ciliates was recorded. In all the examinations of the entire study the relative abundance of each ciliate in the stomach, intestine, and rectum was estimated as formerly and the respective densities of population were recorded as "light," "moderate," or "heavy" (Beers, 1948). If dividing individuals of a particular species were present, the number of such individuals, as well as the total number, was counted in samples containing

50–150 ciliates of the species. From these totals the percentage of dividing individuals in the samples was calculated and this figure was assumed to represent conditions in the urchin. The presence of an occasional dividing specimen was ignored. But if one specimen or a larger number was dividing in every 100 individuals of the species, division was judged to be significant and the appropriate percentage was recorded.

After two weeks of starvation, several urchins of each group were again examined and the condition of their ciliates was recorded. Then, smaller numbers of starved urchins (usually 10 to 20) were removed to other aquaria and were supplied with pieces of healthy fronds of the kelp *Laminaria* sp., which is a preferred food of *S. droebachiensis* and is indeed adequate, as Swan (1961) demonstrated, to maintain it in a healthy, growing condition for at least a year. Usually the urchins were placed directly on, or in contact with, a piece of kelp; thus, they sensed its presence at once and began to feed without delay. Since an excess of kelp was maintained in the aquaria, ample food was always available to the urchins. These laminaria-fed urchins were then opened and examined at various hourly intervals following the addition of food. By varying the time of day at which laminaria was initially supplied, examinations could be conveniently planned for any sequence of hourly intervals within the period of experimental feeding, which was arbitrarily restricted to 5 days. By using sufficient numbers of urchins, nearly all the hourly intervals of the 5-day period were finally represented in the results. It is true that the number of urchins examined for any particular hour was relatively small (usually two to five), since the examination of an urchin requires 15–45 minutes, depending on the condition of its ciliates. But the total number of such urchins was considerable and amounted finally to 294.

RESULTS

1. Condition of the ciliates in urchins collected in nature

Thirty-two urchins taken from the various collections and examined immediately showed the usual ciliate distribution and population densities (some "light," but many "moderate" to "heavy"), in agreement with earlier observations (Beers, 1948). Whereas *B. gracilis* was dividing in all the urchins, only one contained dividing specimens of *E. borealis,* and another those of *M. indomita,* though division in each case was sparse. The digestive tracts of the urchins appeared to contain ample food, chiefly masses of filamentous green algae and fragments of laminaria. These observations indicated that the urchins which were subjected to starvation were healthy, adequately fed, and typical with respect to ciliates; that *B. gracilis* was dividing in all of them; but that only about 3% of them contained dividing specimens of *E. borealis,* and a like percentage those of *M. indomita.*

2. Condition of the ciliates in starved urchins

Thirty-five urchins from various experimental groups were examined after 14–21 days of starvation. In all of them the stomach and the greater part of the intestine were empty (lacked solid food); only the terminal quarter of the intestine and the rectum contained some undigested or indigestible material, but the amount was small. *Entodiscus borealis* was present in the intestine and rectum, as well as the stomach, and *M. indomita* was present in the rectum, as well as the intestine. Evidently these ciliates, as digestion proceeded, had dispersed aborally from their preferred sites. In accordance with expectations, dividing specimens of the two were absent and population densities were reduced to "moderate" or "light." (All urchin ciliates escape regularly in limited numbers among the fecal pellets; in the absence of division, reduced numbers are the rule.) As usual, *B. gracilis* was restricted to the rectum, but dividing specimens were absent in all the urchins except one, in which only two such specimens could be found. Microscopic examination of representatives of the three species showed that the cytoplasm was very transparent and contained relatively few gastrioles. Evidently a two-week period of starvation of the host was adequate to reduce the division rate of all the ciliates practically to zero, and it was concluded that the starved urchins contained to all practical purposes no dividing ciliates.

3. *Feeding habits of starved urchins*

The starved urchins fed readily on laminaria. The stomach was well filled after 6–8 hours of feeding; the stomach and intestine after 15–18 hours (counting from the beginning); and the stomach, intestine, and rectum after about 20 hours. Feeding continued (by night as well as by day) for 60–72 hours, when many of the urchins evidently became surfeited and moved away from the food. On the fourth and fifth days of the experiment, some urchins were always feeding, while others rested near-by on the sides of the aquarium. Thus, feeding occurred irregularly on these days. Some of the urchins were kept a total of 6 to 14 days with the laminaria—well beyond the end of the formal experimental period. These urchins also fed irregularly, probably as urchins feed under natural conditions. The intestine and rectum were always well filled, but the amount of food in the stomach was variable.

4. *Condition of the ciliates in urchins fed laminaria after two weeks without food*

The results of the feeding experiments are summarized in Table I. It is understood that this table is a composite of all experiments, rather than a continuous record of a single vast experiment. The table concerns 294 urchins (total of Column 2), and it is obviously impossible for one investigator to start with this number of starved urchins and to continue the examinations uninterruptedly for 120 hours. In the table the successive hours are grouped by 8-hour periods, and for reference purposes the periods are numbered (Column 1). It is important to note that all the urchins of any period are represented in each of the three columns under "Number (and percentage) of urchins. . . ." A consideration of some of the periods will clarify the method of presentation. When two paragraphs appear under a period in the following account, the first deals with results presented in the table, and the second contains explanatory or supplementary comments.

Period 1. There was examined a total of 20 urchins (Column 2) that had fed from 1 hour to 8 hours on laminaria (Column 3). Since no dividing ciliates could be found in any of these urchins, it follows that the remaining columns of the period read zero.

Period 2. In this period 30 urchins that had fed from 9 to 16 hours were examined. In 18 of the 30, or 60% of them, *E. borealis* was dividing (Column 4), but dividing specimens were absent in the remaining ciliates.

The number of dividing specimens of *E. borealis* in the 18 urchins usually amounted to 4–8% of the total, but in some urchins the number attained 15–20%. For example, on July 11 ten samples were examined from the stomach of a 51-mm. urchin that had fed for 10 hours. They contained a total of 522 individuals of *E. borealis,* of which 88 (nearly 17%) were judged by either of two criteria to be dividing: an elongated condition of the macronucleus or the presence of a transverse cytosomal constriction. Such specimens are easily recognized in living material, even with magnification as low as 10–20 ×, for *E. borealis* is a large ciliate, measuring on the average 143 μ × 87 μ (Powers, 1933a). Many additional individuals were evidently preparing to divide, in view of their large size (length, 160–180 μ), and many had already divided, judged by the great variation in their size (length, 120–170 μ). (The individuals of stable, non-dividing populations of *E. borealis* are remarkably uniform in size.) Microscopic examination of some of the specimens showed that they had lost much of their transparency and contained great numbers of gastrioles. It is a fact that this single urchin contained more dividing individuals of *E. borealis* than I had seen in all the hundreds of urchins examined in three earlier summers. In a 42-mm. urchin examined on August 16 after 15 hours of feeding, 20% of the individuals of *E. borealis* were dividing.

Period 3. Nearly half the urchins (48%) contained dividing specimens of *E. borealis*, but *M. indomita* remained non-divisional. However, the division of *B. gracilis* was resumed in 40% of the urchins.

TABLE I

Incidence of division of three species of ciliates in urchins (Strongylocentrotus droebachiensis) which were starved for two weeks and then fed generously on Laminaria during a 5-day experimental period

Successive 8-hour periods	No. of urchins examined per 8-hour period	Time in hours after beginning of feeding	Number (and percentage) of urchins in which ciliate indicated was dividing		
			Entodiscus borealis	*Madsenia indomita*	*Biggaria gracilis*
1	20	1–8	0	0	0
2	30	9–16	18 (60)	0	0
3	25	17–24	12 (48)	0	10 (40)
4	21	25–32	10 (48)	0	17 (81)
5	19	33–40	9 (47)	0	19 (100)
6	22	41–48	9 (41)	0	22 (100)
7	17	49–56	7 (41)	6 (35)	17 (100)
8	24	57–64	19 (79)	23 (96)	24 (100)
9	20	65–72	13 (65)	13 (65)	20 (100)
10	18	73–80	6 (33)	12 (67)	18 (100)
11	21	81–88	6 (29)	10 (48)	21 (100)
12	24	89–96	4 (17)	6 (25)	24 (100)
13	13	97–104	2 (15)	3 (23)	13 (100)
14	10	105–112	1 (10)	2 (20)	10 (100)
15	10	113–120	2 (20)	2 (20)	10 (100)

The first dividing specimens of *B. gracilis* appeared in one of four urchins that had fed for 21 hours. Of 12 urchins that had fed for 22, 23, or 24 hours, *B. gracilis* was dividing in nine. Although *B. gracilis* is also a fairly large ciliate in which dividing specimens are easy to recognize, the number of such specimens was never great in any urchin of the entire study; usually it amounted to 2–4% of the total. Evidently division is resumed in *B. gracilis* soon after the arrival of fresh food in the rectum.

Periods 4–6. The division of *E. borealis* continued in many of the urchins (41–48%), but *M. indomita* was still not dividing. In Period 4, dividing specimens of *B. gracilis* were present in 81% of the urchins; in Periods 5 and 6, and indeed in all subsequent periods, it was dividing in all the urchins.

In an ideal experiment the history of each of the ciliates should be followed in one and the same urchin by removing samples at intervals throughout the experiment. Unfortunately, this procedure is not feasible at present, and an urchin must be sacrificed at each examination. Such an examination may not reveal correctly the actual physiological condition of the ciliates with respect to division. For example, reference to Table I (Period 4, Column 4) shows that *E. borealis* was dividing in 10 of the urchins; it is understood that it was not dividing in the remaining 11 at the time of the examinations. But in some of the 11, there was great variation in size among the individuals and they contained many gastrioles, suggesting that they had already divided at least once and were preparing for another division. Thus, in many urchins in which *E. borealis* was recorded as non-divisional, it was probably in a cycle of division and the percentages recorded in the table are actually low. These same comments apply to *M. indomita,* beginning with Period 7.

Period 7. This period is of special interest, in that dividing specimens of *M. indomita* made their first appearance in many of the urchins (35%).

The first dividing specimens of *M. indomita* appeared in a 49-mm. urchin that had fed for 50 hours, although they amounted to only 1% of the total. The division of *M. indomita* offered special difficulties of observation, since it is a slender, flattened, transparent ciliate, which is very active in samples of enteric fluid diluted with sea water for making counts. Magnifications high enough to reveal the condition of the macronucleus in living specimens (about 45 ×) render especially difficult the counting of specimens in samples. Therefore, the presence of a cytosomal constriction was used as the sole criterion of division and many pre-divisional specimens with elongated macronuclei were undoubtedly overlooked. The number of dividing individuals identified and recorded by the method never exceeded 5%.

Period 8. In this period the division of *E. borealis* and *M. indomita* attained its maximal incidence in the urchins (79% and 96%, respectively).

It is scarcely necessary to mention that population densities increased measurably in all the ciliates as division continued.

Periods 9–15. In these periods the percentage of urchins that contained dividing specimens of *E. borealis* and *M. indomita* gradually decreased, but with minor fluctuations which may have resulted from variations in the total numbers of urchins examined.

In urchins kept four days or longer with food, it is somewhat difficult to relate satisfactorily the division of *E. borealis* and *M. indomita* to the amount of food in the gut, since many urchins stop feeding after three days and then feed irregularly. Sixteen urchins which had remained 9–14 days with food were examined. Two had dividing specimens of *E. borealis* and one had those of *M. indomita*. Conditions in these urchins probably approximated those found in urchins under natural conditions; that is, the amount of food in the gut was variable and the condition of the two ciliates was unpredictable, though usually non-divisional.

DISCUSSION

The results show conclusively that under certain experimental conditions (starvation of the host, followed by generous feeding) there is a direct relation between the feeding of the urchin and the division of its ciliates: starved urchins contain no dividing ciliates, whereas many urchins fed on laminaria contain great numbers. With reference to the time at which division is resumed in experimentally fed urchins, the results permit these conclusions: division is resumed in *B. gracilis* after 20–30 hours of feeding; a period of division is initiated in *E. borealis* after 10–15 hours of feeding; a similar period is initiated in *M. indomita* after 50–60 hours. The evidence indicates that division continues indefinitely in *B. gracilis*, provided any appreciable amount of food remains in the rectum. The duration of the period of division of *E. borealis* and *M. indomita* is difficult to determine, owing to irregularities in the feeding habits of the host; the evidence indicates that it continues for two to three days and then subsides in most of the urchins.

It is clear that the results cast serious doubt on my earlier postulate to the effect that cycles of division are inherent in *E. borealis* and *M. indomita,* though they do not actually disprove the existence of such cycles. Nevertheless, it seems more likely that the occasional outbreaks of divisional activity are associated with periods of generous feeding on the part of the host.

For the present the precise factors that are responsible for the division of the ciliates must remain unidentified. Presumably the presence of abundant food in the urchin gut results in a great increase in the numbers of bacteria that are both available and suitable as food for the ciliates. There is no doubt that the bacterial flora is greatly augmented by generous feeding on the part of the host. An examination of enteric fluid from a well-fed urchin reveals great numbers of bacteria, whereas fluid from a starved urchin contains relatively few. It is recognized that absolute numbers may be of little significance, for some ciliates are extremely selective in their ingestion of bacterial food. For example, Kidder (1941, p. 471) isolated from a mass culture of *Tillina canalifera* 26 types of bacteria, only one of which was suitable to maintain the growth of the ciliate. Nevertheless, the many gastrioles in ciliates from well-fed urchins stand as proof of generous feeding, and the division of the ciliates has been conclusively demonstrated. Thus, it is clear that suitable bacteria were present in plentiful numbers, and it is assumed that division was the direct result of increased food ingestion. Whether the digestive juices of the urchin are important in stimulating division is unknown.

Until the dietary requirements of the three ciliates are conclusively established, the facts seem to permit the following interpretation of the normal urchin-ciliate relationship. Ingested material is nearly always present in the rectum of any urchin collected in nature, and this material suffices to support a bacterial flora that is both adequate and suitable to maintain *B. gracilis* in a constantly dividing state. The amount of ingested material in the stomach and intestine of such an urchin is demonstrably variable. The flora that it supports is considerable, but usually the flora suffices merely to maintain *E. borealis* and *M. indomita* in a non-dividing state. Occasionally an urchin finds a plentiful supply of food and fills to repletion. Then the numbers of suitable bacteria increase to the extent that they are adequate to initiate and sustain a period of division.

SUMMARY

1. The study deals with division in the ciliates *Entodiscus borealis, Madsenia indomita,* and *Biggaria gracilis.* All the urchins collected in nature contained dividing specimens of *B. gracilis,* but only 3% of them contained those of *E. borealis* and *M. indomita.*

2. Urchins were kept without food for 2–3 weeks; in these, dividing specimens of the ciliates were absent.

3. After about two weeks of starvation, the urchins were supplied with generous amounts of the kelp *Laminaria,* and the percentage of the urchins that contained dividing specimens of each of the ciliates was recorded by successive 8-hour periods during 5 days of feeding.

4. Division began in *E. borealis* after 10–15 hours of feeding by the host, and dividing individuals were present for about three days in 33–79% of the urchins.

5. Division began in *M. indomita* after 50–60 hours, and dividing specimens were present for about two days in 25–96% of the urchins.

6. Division began in *B. gracilis* after 20–30 hours, and dividing specimens were present in all the urchins after the second day.

7. Division appears to continue indefinitely in *B. gracilis,* provided any appreciable amount of food is present in the urchin gut. Although division appears to occur discontinuously in *E. borealis* and *M. indomita,* it is doubtful that cycles of division are inherent in them, as postulated earlier. It seems more likely that their division is correlated with the copious ingestion of suitable bacteria, whose numbers are greatly increased by the presence of abundant food in the urchin gut.

LITERATURE CITED

Beers, C. D., 1948. The ciliates of *Strongylocentrotus droebachiensis:* Incidence, distribution in the host, and division. *Biol. Bull.,* **94**: 99–112.

Beers, C. D., 1961. The obligate commensal ciliates of *Strongylocentrotus droebachiensis:* Occurrence and division in urchins of diverse ages; survival in sea water in relation to infectivity. *Biol. Bull.,* **121**: 69–81.

Kidder, G. W., 1941. The technique and significance of control in protozoan culture. *In:* Calkins and Summers: Protozoa in Biological Research, pp. 448–474. New York: Columbia University Press.

Powers, P. B. A., 1933a. Studies on the ciliates from sea urchins. I. General taxonomy. *Biol. Bull.,* **65**: 106–121.

Powers, P. B. A., 1933b. Studies on the ciliates from sea urchins. II. *Entodiscus borealis* (Hentschel), (Protozoa, Ciliata), behavior and morphology. *Biol. Bull.,* **65**: 122–136.

Swan, E. F., 1961. Some observations on the growth rate of sea urchins in the genus *Strongylocentrotus. Biol. Bull.,* **120**: 420–427.

BIOLOGICALLY ACTIVE PEPTIDES FROM THE PERICARDIAL ORGANS OF THE CRAB CANCER BOREALIS[1]

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Pericardial organs are neurosecretory structures located in the pericardial cavity of decapod and stomatopod crustaceans. Alexandrowicz (1952, 1953) was the first to figure these structures and, with Carlisle, to show that low concentrations of aqueous extracts acted as a potent cardio-excitor (Alexandrowicz and Carlisle, 1953). Extracts produced a marked increase in amplitude and frequency in all the crustacean species studied except *Maia squinado,* which exhibited an increase in amplitude, but a decrease in frequency. These investigators also found that blood collected from the pericardial cavity produced excitation similar to that of pericardial organ extracts. Cooke (unpublished data) has demonstrated that cardio-excitor hormone is released by electrical stimulation of the pericardial organs.

Carlisle (1956) reported finding two active areas when pericardial organ extracts were subjected to paper chromatography, both of which gave color reactions indicative of indole alkylamines. One of the active areas was destroyed by ortho-diphenol oxidase more rapidly than the other, an action that led Carlisle to believe the latter to be a precursor. Carlisle speculated that the active substance might be an ortho-dihydroxytryptamine, and later considered 5,6-dihydroxytryptamine the most likely possibility (Carlisle and Knowles, 1959).

Maynard and Welsh (1959) found 5-hydroxytryptamine to be present in pericardial organ extracts, but at concentrations too low to account for the marked activity of the extract. These authors demonstrated that pericardial organ extracts could be inactivated by trypsin and chymotrypsin, and that the active principle was (1) dialyzable, (2) soluble in aqueous and alcoholic solvents, but only sparingly soluble in acetone, and (3) heat-stable at neutral and acid pH. They tentatively identified the neurosecretory material as a peptide.

The purpose of this paper is to present further data from studies on the nature of the pericardial organ neurohormone.

MATERIALS AND METHODS

Cancer borealis and *Cancer irroratus* were used as a source of pericardial organs; lobster hearts were used for assay. Animals were obtained from the Crab and Lobster Company, Boothbay Harbor, Me., or from local sources. Pericardial organs were removed by the method of Maynard and Welsh (1959) and either used

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immediately or freeze-dried and stored in a desiccator at 10° C. Pericardial organs stored by this method for more than one year were still active.

Extracts of pericardial organs were made by grinding either the fresh or freeze-dried tissue in a glass homogenizer with the appropriate solvent. Aqueous extracts were heated at 100° C. for a few minutes and centrifuged, the sediment being washed several times and the washes pooled with the supernatant. For paper chromatography or paper electrophoresis, extracts were generally made in 95% ethanol and washed with petroleum ether.

All extracts not made in *Homarus* perfusion fluid (Welsh and Smith, 1960, after Cole) were lyophilized or taken to dryness under reduced pressure. The residue was redissolved in perfusion fluid before assaying. Extracts were tested on a perfused isolated lobster heart which was kept at a constant temperature. Although individual experiments were done at constant temperatures, the range of temperatures during the course of these experiments was 15° to 20° C. A small volume (0.5 ml.) of the extract was introduced into the perfusion medium by syringe without disturbing the rate of flow of the fluid, and the action of the heart was recorded on a smoked drum.

Paper electrophoresis of pericardial organ extracts was carried out utilizing Whatman 3 MM paper and buffers (acetate, phosphate, Tris, borate) with a pH range of 2.6 to 10.1 and ionic strength of 0.05. Voltage was usually 11.4 V./cm., although runs were also made at 5 V./cm. The extract, from 1–10 pairs of pericardial organs, was taken down to dryness under reduced pressure. It was then redissolved in a small amount (50–100 μl.) of 95% ethanol and streaked at the center of a prewashed paper. Lengths of run were 1 to 24 hours, after which papers were either air-dried, oven-dried at 60° C. and cut into sections, or cut while still wet. The latter method was the method of choice. The section which included the origin and one section to either side of the origin was of 1 cm. width, while all other sections were 2 cm. The sections were eluted into 3 ml. of solvent, freeze-dried if run in volatile buffers, and assayed. For staining, strips were cut from the long axis of the paper before sections were made.

Chromatography of pericardial organ extracts was performed by the ascending one-dimensional method, utilizing n-butanol/acetic/water (60:15:25) and Whatman 3 MM paper. Papers were pre-washed with distilled water and ethanol. Development was usually 12–17 hours at room temperature. An extract from 4 to 20 pairs of pericardial organs was applied as a streak and developed, after which a longitudinal strip was cut from the paper and sprayed with various staining reagents. In this way the area of biological activity was correlated with staining reaction.

Eluates of the areas which produced activity and which were ninhydrin-positive were hydrolyzed by the method of Consden, Gordon and Martin (1947). The hydrolysates were spotted on Whatman No. 1 paper, along with amino acid standards, developed in n-butanol/acetic/water (60:15:25) or n-butanol/pyridine/water (1:1:1). All hydrolysis experiments were done with pericardial organs from *Cancer borealis*.

The confirmation of acidic and basic amino acid residues was accomplished with paper electrophoresis employing Schleicher and Schuell number 589 paper and 0.05 *M* acetate buffer at pH 4.7. The hydrolysate, after being dried over NaOH, was taken up in dilute NH_4OH and applied to the center of the paper. A paper with lysine, glycine and glutamic acid applied at the origin was run simultaneously with the hydrolysate as a standard.

RESULTS

Inactivation of pericardial organ active substance was accomplished by incubating extracts made in *Homarus* perfusion fluid (pH 7.6) with crystalline trypsin or chymotrypsin (approximately 10 μg./ml. extract) for one hour at 37° C. The enzymes alone had little, if any, effect on the heart. Heated enzyme incubated with pericardial organ extract did not affect the activity. These results confirm those obtained by Maynard and Welsh (1959). Trypsin was used periodically to inactivate extracts and eluates from paper electrophoresis and chromatography. This gave assurance that the activity was due to pericardial organ cardio-excitor substance.

A typical assay of a pericardial organ extract after electrophoresis is shown in Figure 1. As was true in most assays, the activity was spread over several centimeters with a major peak at one fraction. Where activity was found to be equally distributed between fractions, it is assumed that the fraction boundary line cut the activity peak in two.

The data in Figure 2 demonstrate that the isoelectric point of the active material is near pH 3, and an increase in pH above this point causes a corresponding increase

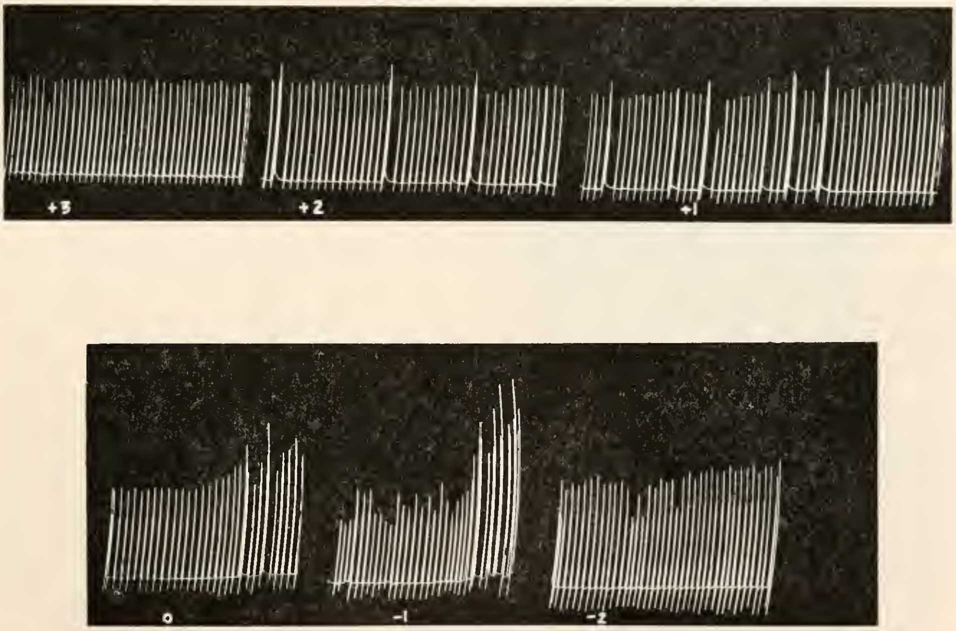

FIGURE 1. A typical assay of pericardial organ extract after paper electrophoresis. Extract of four pairs of *Cancer borealis* pericardial organs. Each fraction eluted into 3.0 ml. perfusion fluid and assayed on a perfused isolated lobster heart. Tris buffer, pH 7.7, 14 hours, 150 V.

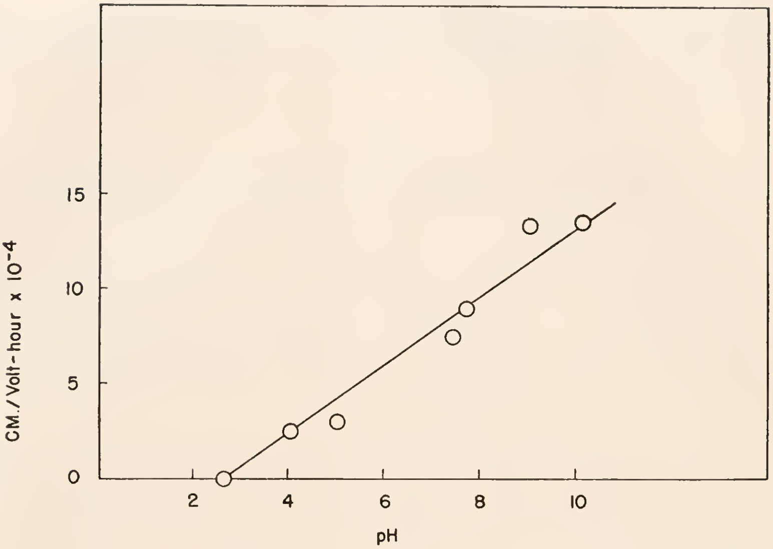

FIGURE 2. Isoelectric point of pericardial organ cardio-excitor material. Buffer ionic strength 0.05.

in migration toward the anode. A series of electrophoresis runs at pH 9.0, of increasing lengths of time, was made in an attempt to correlate staining reaction with migration of activity. Of the numerous reagents applied, only ninhydrin consistently stained the active area.

Tests of eluates of chromatogrammed pericardial organ extracts showed activity from two areas. This activity resembled but did not equal that produced by crude extracts. Such activity could be inactivated by trypsin. The activity was localized to two ninhydrin-positive areas (Fig. 3).

Chromatography of acid hydrolysates of the two active areas showed that these were two peptides of similar nature. These peptides have been given the designation peptides A and B. Hydrolysates of these peptides were subjected to paper chromatography and paper electrophoresis under similar conditions. These revealed the presence of glutamic acid and a positively charged amino acid (lysine or arginine), as well as the presence of neutral amino acids. Based on staining intensity, the glutamic acid was present in greater concentration than the positively charged amino acid. One other acidic residue, which had a migration rate slightly higher than that of glutamic acid, was also present in low concentration (Fig. 4). The neutral amino acids were categorized by their R_f values and comparison with standards in two chromatographic solvents, and were tentatively identified as serine in both peptides, tyrosine in peptide A, and methionine in peptide B. Each peptide contained one other residue which appeared in the region of valine or phenylalanine.

In order to demonstrate that one was dealing with the same material in electrophoresis and chromatography, a pericardial organ extract was subjected to electrophoresis and the area of activity determined by assay. A matching area from a simultaneously run paper was then eluted and subjected to paper chromatography. Examination showed that the two active, ninhydrin-positive areas present in the

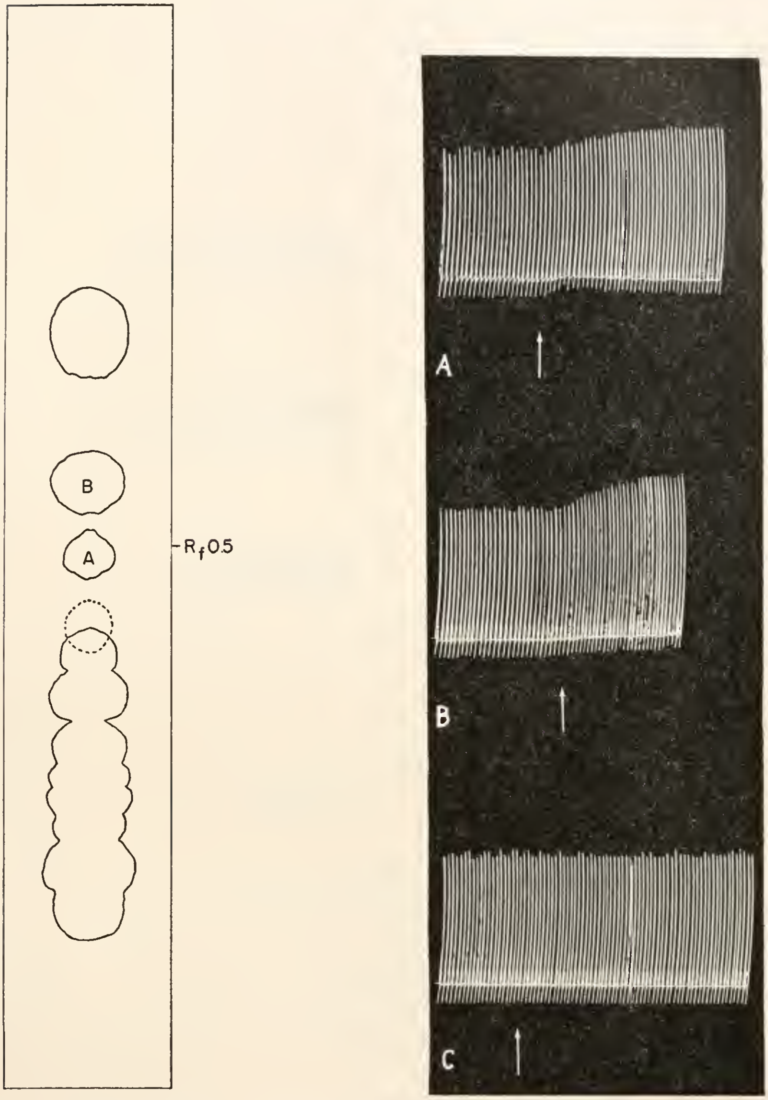

FIGURE 3. Chromatography and assay of an extract of 20 pairs of pericardial organs; n-butanol/acetic/water; 14 hours. Ninhydrin stain. A and B are active areas, C is control. Arrow marks point at which eluate reaches heart.

chromatogrammed pericardial organ extract were also present in eluates of active areas following electrophoresis.

DISCUSSION

Maynard and Maynard (1962) estimate that the neurosecretory granules may form 2% or less of the volume of the trunks of brachyuran pericardial organs. Since the average dry weight of freeze-dried pericardial organs from *Cancer borealis* was approximately 0.6 mg./animal, the amount of material in the neurosecretory

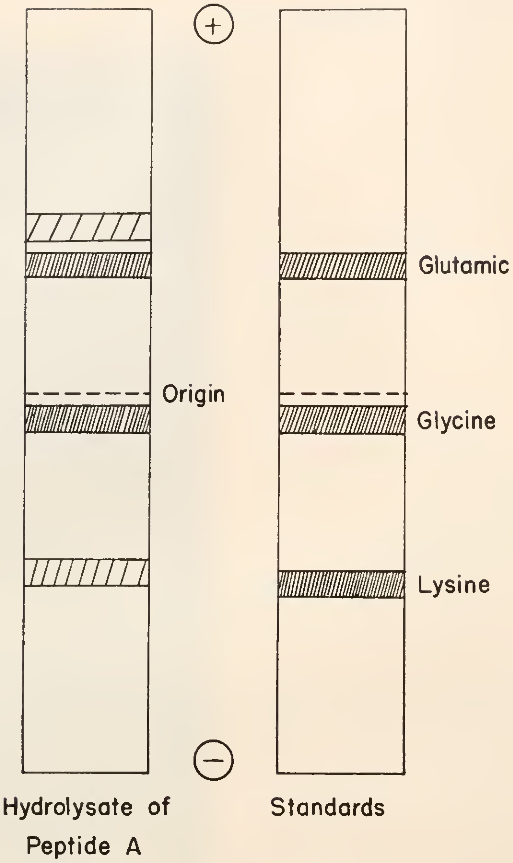

FIGURE 4. Electrophoresis of hydrolysate of peptide A. Acetate buffer, pH 4.7, ionic strength 0.05, 200 V., 4 hours.

granules can be calculated to be approximately 12 μg./animal. Although there is disagreement as to whether neurohormones are attached to a carrier protein or whether they are split off from a parent molecule (Bern, 1962), large protein molecules appear to be present in most, if not all, neurosecretory granules. The presence of such a protein, along with other components, would make the figure for actual neurohormone material present in the pericardial organ somewhat less than the calculated 12 μg./animal.

Pericardial organ extracts subjected to paper electrophoresis and paper chromatography apparently lose some activity. Smyth (1959) also noted this with crayfish pericardial organs, and found that developing chromatograms in an atmosphere of nitrogen or in the presence of ascorbic acid did not prevent loss of activity. With paper electrophoresis there was a correlation between the length of run and activity recovered, *i.e.,* short runs retained proportionally more activity.

The fact that there are two active components is not surprising. Carlisle (1956) reported finding two areas of activity after chromatography of pericardial organ extract. Of interest in this respect is the report by Alexandrowicz (1953) in which he distinguishes two types of neurosecretory fibers contributing to the pericardial organ system of *Squilla*. Electron microscopy (Knowles, 1960) shows two types of neurosecretory granules in pericardial organs of *Squilla,* differing in size and internal structure. More recently, Maynard (1961) demonstrated the presence of three types of secretory cells contributing fibers to the pericardial organ-anterior ramification complex in several species of decapod crustaceans. When examined by electron microscopy, *Cancer* pericardial organs showed neurosecretory granules of only one size, but the other brachyuran genera examined contained granules of two or three size groups (Maynard and Maynard, 1962).

The data indicate that the active compounds extracted from pericardial organs are acidic peptides with an isoelectric point near pH 3. The presence of proportionally more glutaminyl residues than positively charged residue (lysine or arginine) fits well with this conclusion. The proposed amino acid composition of the peptides fits the requirements for enzymatic degradation by trypsin and chymotrypsin, since trypsin is specific for lysine or arginine linkages, while chymotrypsin is specific for aromatic, as well as leucyl, methionyl, arginyl, and glutaminyl bonds.

The identification of the neutral and basic amino acid residues has been made on a tentative basis. An examination of peptides A and B at this time indicates the possibility of a single amino acid difference. A small difference, especially in the neutral amino acids, would account for the inability to separate the peptides by means of paper electrophoresis. The residue exhibiting slightly higher negative charge than glutamic acid has not been identified. It is possible that a non-amino acid residue is present with the peptide. Smyth (1959) noted that after chromatography of crayfish pericardial organs in methanol/water/pyridine the active area stained lightly with ammoniacal silver nitrate as well as with ninhydrin.

Tryptophan analysis was not feasible because of the small amount of active material present in pericardial organs. However, it should be pointed out that pericardial organ extracts and 5-hydroxytryptamine have a qualitatively similar action on the decapod heart (Maynard and Welsh, 1959; Cooke, 1962), and this similarity may be due to the presence of tryptophan in the peptide.

Carlisle has considered 5,6-dihydroxytryptamine as the most likely possibility for the pericardial organ neurohormone. Such a compound has been shown to be extremely labile, exhibiting spectral shifts after 30 minutes in aqueous solution at pH 7, and after 5 minutes at pH 8 (Schlossberger and Kuch, 1960). There have been no published data to show that such a compound, in fact, exists in pericardial organs of decapod crustaceans, although the possibility is not discounted. If present, it may be acting at a different site on the cardiac ganglion, as has been demonstrated for 5-hydroxytryptamine (Cooke, 1962). The rate at which pericardial organ extracts used in this study lose activity, as well as other criteria, indicates that the activity of these extracts is not due entirely to 5,6-dihydroxytryptamine.

It is interesting to note that enzyme inactivation studies of crustacean chromatophorotropins and retinal pigment hormones show that all these neurosecretory products are inactivated by either trypsin or chymotrypsin or both of these enzymes (Knowles, Carlisle and Dupont-Raabe, 1956; Pérez-González, 1957; Kleinholz, Esper, Johnson and Kimball, 1961). It is well known that the neurosecretory hormones of the vertebrate hypothalamic-neurohypophyseal system are peptides. Future research may show that one of the main functions of neurosecretory systems is the production of biologically active peptides.

I wish to express my appreciation to Professor John H. Welsh for his continued guidance and interest in this problem, to Dr. Russell F. Doolittle for his invaluable advice, and to Misses Carolyn Sharpe and Joyce Zipf for help in the dissections.

Summary

1. The isoelectric point of pericardial organ cardio-excitor material, determined by paper electrophoresis, is near pH 3.

2. Paper chromatography demonstrates two active compounds in pericardial organ extracts which stain with ninhydrin and upon acid hydrolysis are identified as peptides. These peptides differ in composition by only a small number of residues.

3. The peptides contain proportionally more glutaminyl residues than positively charged residue (lysine or arginine), one other negatively charged residue, and a small number of neutral amino acids.

LITERATURE CITED

Alexandrowicz, J. S., 1952. Notes on the nervous system in the stomatopods. I. The system of median connectives. *Pubbl. Staz. Zool. Napoli,* **23**: 201–214.

Alexandrowicz, J. S., 1953. Nervous organs in the pericardial cavity of the decapod Crustacea. *J. Marine Biol. Assoc.,* **31**: 563–580.

Alexandrowicz, J. S., and D. B. Carlisle, 1953. Some experiments on the function of the pericardial organs in Crustacea. *J. Marine Biol. Assoc.,* **32**: 175–192.

Bern, H. A., 1962. The properties of neurosecretory cells. *Gen. Comp. Endocrin., Supplement,* **1**: 117–132.

Carlisle, D. B., 1956. An indole-alkylamine regulating heart-beat in Crustacea. *Biochem. J.,* **63**: 32–33 P.

Carlisle, D. B., and F. Knowles, 1959. Endocrine Control in Crustaceans. Cambridge University Press, Cambridge.

Consden, R., A. H. Gordon and A. J. P. Martin, 1947. The identification of lower peptides in complex mixtures. *Biochem. J.,* **41**: 590–596.

Cooke, I., 1962. Effects of the pericardial organ neurosecretory substance on the crustacean heart. *Gen. Comp. Endocrin.,* **2**: 29.

Kleinholz, L. H., H. Esper, C. Johnson and F. Kimball, 1961. Characterization and partial purification of crustacean eyestalk hormones. *Amer. Zool.,* **1**: 366.

Knowles, F. G. W., 1960. A highly organized structure within a neurosecretory vesicle. *Nature,* **185**: 709–710.

Knowles, F. G. W., D. B. Carlisle and M. Dupont-Raabe, 1956. Inactivation enzymatique d'une substance chromactive des insectes et crustacés. *C. R. Acad. Sci., Paris,* **242**: 825.

Maynard, D. M., 1961. Thoracic neurosecretory structures in Brachyura. II. Secretory neurons. *Gen. Comp. Endocrin.,* **1**: 237–263.

Maynard, D. M., and E. A. Maynard, 1962. Thoracic neurosecretory structures in Brachyura. III. Microanatomy of peripheral structures. *Gen. Comp. Endocrin.,* **2**: 12–28.

Maynard, D. M., and J. H. Welsh, 1959. Neurohormones of the pericardial organs of brachyuran Crustacea. *J. Physiol.,* **149**: 215–227.

Pérez-González, M. D., 1957. Evidence for hormone-containing granules in sinus glands of the fiddler crab *Uca pugilator.* *Biol. Bull.,* **113**: 426–441.

Schlossberger, H. G., and H. Kuch, 1960. Synthese des 5,6-Dihydroxy-tryptamins. *Chem. Ber.,* **93**: 1318–1323.

Smyth, T., Jr., 1959. Paper chromatography of pericardial organs of crayfish. *Biol. Bull.,* **117**: 426.

Welsh, J. H., and R. I. Smith, 1960. Laboratory Exercises in Invertebrate Physiology. Revised Edition. Burgess Publ. Co., Minneapolis.

THE GENETICS OF ARTEMIA SALINA. II. WHITE EYE, A SEX-LINKED MUTATION [1]

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Although there have been many cytological studies of sex determination in the Crustacea (reviewed by Niiyama, 1959), this paper will describe the first genetic study of crustacean sex determination. It will be shown that the female is the heterogametic sex in the brine shrimp. Sex differentiation in *Artemia* has been discussed by Bowen and Hanson (1962).

The culture medium and the genetic techniques used in the following experiments were described in the first paper in this series (Bowen, 1962).

The author would like to thank Miss Jean Hanson, Mr. Dan Straus, and Mr. H. Stuart Williamson for their assistance throughout this study.

MATERIALS

Origin of stock #5

In all the races of *Artemia*, the wild-type eye is black. The recessive autosomal gene, *r*, for red eyes arose as a spontaneous mutation in the Utah race. It was described in the first paper in this series. A second autosomal gene, *c*, which determines "crinkle eyes," has not been described previously. This mutation arose spontaneously in the California race (San Francisco Bay) and was discovered by Miss Jean Hanson in 1960. At the age of three to five weeks, shrimp of the *cc* genotype develop an extra patch of pigment cells on the eyestalk. The two mutations have been combined in a single line, designated as stock #5 in our laboratory. Young shrimp in this *rr cc* stock have red eyes. At sexual maturity their eyes turn brown or black. At the age of five weeks the pigment in the normal eye field is black, but the pigment in the "crinkles" patch on the eyestalk is still red.

Origin of the white eye mutation

A wild-type male from a cyst collected at Great Salt Lake, Utah, was mated to a female of the #5 stock. An F_1 male with the *RrCc* genotype was backcrossed to another #5 stock female. In this progeny the author found one white-eyed male in December of 1961.

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[1] This research was supported by a grant from the National Science Foundation (NSF G-23863).

RESULTS

The terms *pigmented* or *wild-type phenotype* are used below to designate non-white-eyed shrimp. Because the first white-eyed male had the genotype *Rr,* some of the pigmented shrimp were red-eyed and some were black-eyed in Experiments A, B, and C.

Experiment A

The first white male was mated to a female from the #5 stock. This mating is represented as Generation I in Figure 1. Three broods of pigmented F_1 nauplii were produced: a total of 14 males and 19 females lived to maturity. The F_1 shrimps were mated to each other to produce an F_2 which consisted of 14 white males, 16 pigmented males, and 32 pigmented females (shown in Generation III of the pedigree). In this first experiment, the mode of inheritance of white eye in *Artemia* resembled that of white eye in *Drosophila.*

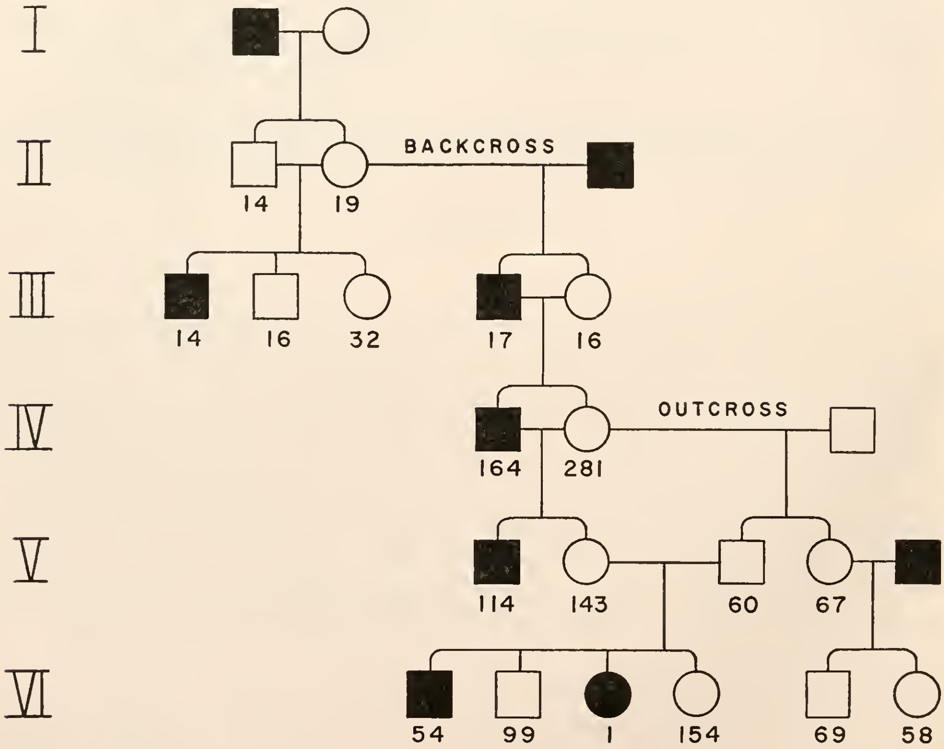

FIGURE 1. Pedigree showing the distribution of white eye in the first six generations. Conventional genetic notation is used: solid symbols indicate shrimps with mutant phenotype; open symbols indicate shrimps with wild-type phenotype (pigmented eyes). Squares represent males; circles represent females. The number written under each symbol indicates the number of progeny in that class.

Experiment B

An F_1 female from Experiment A was backcrossed to the first white male. Surprisingly, the progeny consisted of 17 white males and 16 pigmented females (shown in Generation III of the pedigree in Figure 1). These shrimp were mated *inter se* and produced 164 white males and 281 pigmented females. These were mated to each other and the progeny consisted of 114 white males and 143 pigmented females. Thus, the mating of a white male to the pigmented daughter of a white male results in a pure-breeding stock of white males and pigmented females. This stock is carried in our laboratory as stock #9.

Experiment C

Three females from stock #9 were outcrossed to wild-type males which had hatched from cysts collected in Quemado, New Mexico (U.S.A.). The F_1 shrimps (shown in Generation V of the pedigree) were all pigmented: 60 males and 67 females. Six of the F_1 females were mated to #9 stock white males, and again the progeny were all pigmented: 69 males and 58 females. Four of the F_1 males were mated to females from the #9 stock. These four matings produced 54 white males, 99 pigmented males, 154 pigmented females, and one white female (shown in the sixth generation of the pedigree). This first white female appeared in May of 1962. She was mated to a white brother and produced all white-eyed progeny.

The Hypothesis of Partial Sex Linkage

The experimental results can be accounted for by the following assumptions: (1) The mutant gene *w* which determines white eyes is recessive to its wild-type allele *W*. (2) The females are heterogametic. The chromosome constitution of the female will be represented as XY; the males will be XX. (3) The white locus is partially sex-linked. Because it is on the homologous segment of the sex chromosomes, both males and females may be *WW*, *Ww*, or *ww*.

The first white female probably arose as the result of a crossover between the white locus and the "sex locus." It is not known whether sex is determined by two alleles at one true sex locus or by several loci on the differential segment of the X or the Y chromosome. If there were several loci governing sex determination, the "sex locus" would be designated as the place where the differential segment of a sex chromosome joins the homologous segment (Fig. 2). Both concepts of a sex locus would be in accord with the experimental results.

The cytological studies of *Artemia* (reviewed by Barigozzi, 1957) have not revealed the presence of a pair of chromosomes of unequal length. For this reason the X and Y chromosomes in Figure 2 are shown to be the same length. The length of the differential segment in relation to the homologous segment is an arbitrary choice. Only one differential segment is shown on each sex chromosome although in many species two such segments have been found (reviewed by Darlington, 1958).

Partial sex-linkage has been reported in three genera of viviparous killifishes (family Poeciliidae): in *Aplocheilus latipes* (Aida, 1921), in the guppy, *Lebistes reticulatus* (Winge and Ditlevsen, 1947) and in the platyfish, *Xiphophorus* (*Platy-*

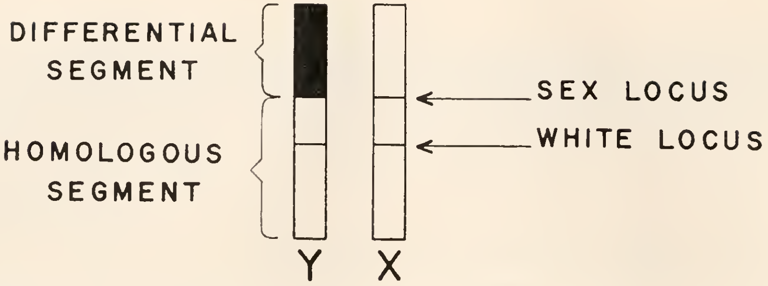
DIFFERENTIAL SEGMENT
HOMOLOGOUS SEGMENT
SEX LOCUS
WHITE LOCUS
Y
X

FIGURE 2. Diagram of the sex chromosomes of *Artemia*. Crossing over may occur between the white locus and the sex locus.

poecilus) *maculatus* (Gordon, 1937, 1947; reviewed by Bellamy and Queal, 1950). Crossover of a gene from the X to the Y chromosome has also been reported at the bobbed locus of *Drosophila melanogaster* (Stern, 1929; Neuhaus, 1937). Although many characteristics in the human have been reported to be partially sex-linked, Morton (1957) has found that in every instance the data fell short of statistical significance.

EXPLANATION OF THE EXPERIMENTAL RESULTS

In Figure 3 the hypothesis of partial sex-linkage is applied to two experiments. On the left is a pedigree which describes Experiment A. If a white male is mated

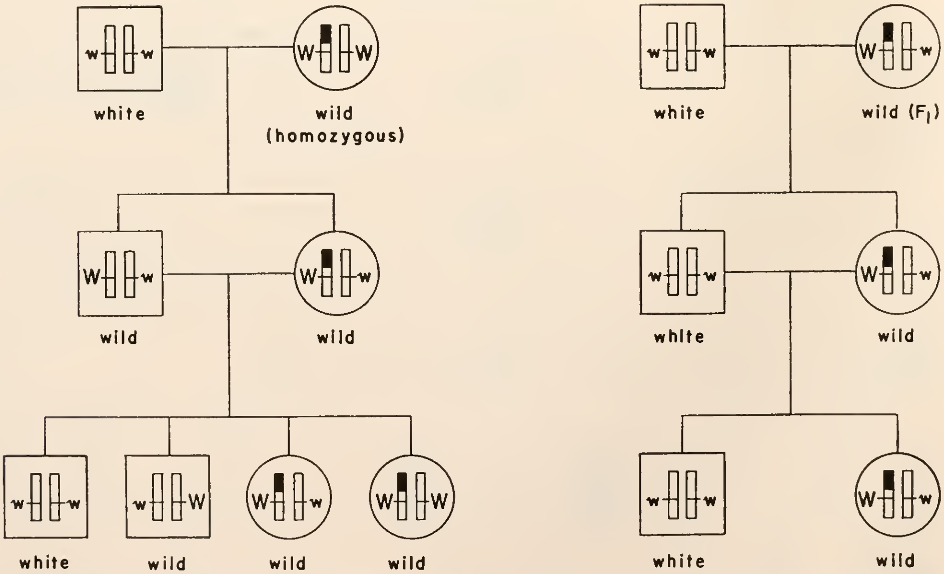
w w
white
W W
wild
(homozygous)
W w
wild
W w
wild
w w
white
w W
wild
W w
wild
W W
wild
w w
white
W w
wild (F1)
w w
white
W w
wild
w w
white
W w
wild

FIGURE 3. Sex-linked inheritance in *Artemia*. Squares represent males; circles represent females. The phenotype of each class is written beneath the symbol. The X and Y chromosomes are shown as in Figure 2. Crossing over between the X and Y does not occur in these two experiments.

to a homozygous wild-type (pigmented) female, all the F_1 progeny will be wild-type. If the F_1 shrimps are mated *inter se,* the F_2 phenotypic ratio would be: ¼ white males: ¼ wild-type males: ½ wild-type females. The observed ratio was 14:16:32.

On the right side of Figure 3 is a pedigree which explains the data in Experiment B. If a white male is mated to the heterozygous daughter of a white male, a pure-breeding stock of white males and phenotypically wild-type females will be established. That there were fewer males than females classified in Experiment B is probably due to the lower viability of the white phenotype (discussed below).

Partial sex-linkage was proposed as the mode of inheritance of white eye before the fourth generation was classified. At that time it was predicted that a white-eyed female would be produced as the result of crossing over. Experiment C was designed to test the hypothesis of partial sex-linkage; the results are in accord with the hypothesis.

SUMMARY OF GENETIC DATA

The data from Experiments A, B, and C, and from additional matings made in the summer of 1962 are combined in Table I. The number of broods may exceed the number of matings because some matings produced more than one brood. The viability of the white phenotype calculated from the progeny of matings of X^wY^W females × X^wX^w males is 623/877 or 0.71. Good agreement is found when the viability is calculated from the all-male progeny of matings of X^wY^W females × X^wX^W males: 99/152 or 0.65. The author is unable to explain the significant deviation from the expected ratio in the progeny of matings of X^wY^W females × X^wX^W males. One would expect the ratio of pigmented females to pigmented males to be 2. The observed ratio is 247/152 or 1.6.

The X and Y chromosomes carried by stock #9 are of special interest. The X chromosome is marked by the mutant gene *w* and was carried by the first white-eyed shrimp (an X^wX^w male). The mutant gene arose spontaneously in the X chromosome which evidently came from the #5 stock. The Y carries the wild

TABLE I

Segregation of the gene w. (*In the genotype of the female parent, the gene on the* Y *chromosome is underlined*)

Number of matings	Number of broods	Mating female × male (XY) (XX)	Number of offspring				
			White male	White female	Pigmented male	Pigmented female	Total
25	32	$W\underline{W}$ × ww	0	0	360	362	722
61	82	$w\underline{W}$ × ww	623	0	0	877	1500
3	4	$w\underline{W}$ × WW	0	0	60	67	127
14	21	$w\underline{W}$ × Ww	99	1	152	247	499
23	31	$w\underline{w}$ × ww	166	174	0	0	340

allele and was present in the #5 female (X^WY^W) to which this male was mated in Generation I. Crossing over between this X^w chromosome and this Y^W chromosome is rare. For example from the #9 stock matings (X^wY^W females × X^wX^w males), a total of 1500 progeny consisted of white males and pigmented females. That is, no crossovers were detected among the 1500 female gametes tested. However, one recombinant was found among the 499 offspring of matings of #9 stock females to X^wX^W males (shown in the fourth line in Table I). Since only one-fourth of the recombinant progeny of such matings can be detected, the crossover rate here is 4/499 or 1/125. The crossover frequency calculated from all the data from #9 females is 1/(125 + 1500) which is 1/1625 or 0.06%.

The racial origin of these two chromosomes is unknown because they originated from the #5 stock which is derived from both the Utah and California races. In a later paper it will be shown that when a female is carrying the same X^w chromosome and a Y^W from another source, the frequency of crossing over may be as high as 9%. Evidently a crossover suppressor mechanism is present in stock #9.

SUMMARY

1. This paper describes the first sex-linked gene discovered among the Crustacea. The experimental results can be accounted for by the following hypotheses: (1) the mutant gene *w*, which determines white eyes, is recessive to its wild allele *W*. (2) Female brine shrimp are heterogametic. The chromosome constitution of the female will be represented as XY; the males are XX. (3) The white locus is partially sex-linked. Because it lies on the homologous segments of the sex chromosomes, both males and females may have the genotype of *WW*, *Ww*, or *ww*.

2. In matings of *Ww* females to *ww* males, crossing over between the white locus and the sex locus may be detected. In the #9 stock, females have the genotype X^wY^W and the frequency of crossing over is low (1/1625 or 0.06%). This results in a "mother-to-daughter" inheritance of the *W* gene in the #9 stock.

LITERATURE CITED

Aida, T., 1921. On the inheritance of color in a fresh-water fish *Aplocheilus latipes* Temmick and Schlegel, with special reference to sex-linked inheritance. *Genetics,* **6**: 554–573.

Barigozzi, C., 1957. Différenciation des génotypes et distribution géographique d'*Artemia salina* Leach: données et problèmes. *Année Biol.,* **33**: 241–250.

Bellamy, A. W., and M. L. Queal, 1950. Heterosomal inheritance and sex determination in *Platypoecilus maculatus. Genetics,* **36**: 93–107.

Bowen, S. T., 1962. The genetics of *Artemio salina.* I. The reproductive cycle. *Biol. Bull.,* **122**: 25–32.

Bowen, S. T., and J. Hanson, 1962. A gynandromorph of the brine shrimp, *Artemia salina. Genetics,* **47**: 277–280.

Darlington, C. D., 1958. The Evolution of Genetic Systems. Basic Books, Inc., New York.

Gordon, M., 1937. Genetics of *Platypoecilus.* III. Inheritance of sex and crossing over of the sex chromosomes in the Platyfish. *Genetics,* **22**: 376–392.

Gordon, M., 1947. Genetics of *Platypoecilus maculatus.* IV. The sex determining mechanism in two wild populations of the Mexican platyfish. *Genetics,* **32**: 8–17.

Morton, N. E., 1957. Further scoring types in sequential linkage tests, with a critical review of autosomal and partial sex linkage in man. *Amer. J. Hum. Genet.,* **9**: 55–75.

NEUHAUS, M. H., 1937. Additional data on crossing over between X and Y chromosomes in *Drosophila melanogaster*. *Genetics,* **22**: 333–339.

NIIYAMA, H., 1959. A comparative study of the chromosomes in decapods, isopods and amphipods, with some remarks on cytotaxonomy and sex-determination in the Crustacea. *Memoirs of the Faculty of Fisheries, (Hokkaido University)*, **7**: 1–60.

STERN, C., 1929. Untersuchungen über Aberrationen des Y-chromosoms von *Drosophila melanogaster*. *Zeitschr. induktive Abstammungs- und Vererbungslehre,* **51**: 253–353.

WINGE, O., AND E. DITLEVSEN, 1947. Colour inheritance and sex determination in *Lebistes*. *Heredity,* **1**: 67–83.

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CHROMATOPHORE CONTROL AND NEUROSECRETION IN THE MUD SHRIMP, UPOGEBIA AFFINIS[1]

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The control of chromatophores in the mud shrimp, *Upogebia affinis,* has not been described. This organism has been used in very few investigations of chromatophores, and in these only as a source of tissues suspected of containing chromatophorotropins. Furthermore, only one account (Hanström, 1937) of the morphology of neuroendocrine structures in this organism is available. In 1948, Hanström summarized the results of his 20-year study of the supraesophageal ganglia and incretory organs in the Malacostraca.

Systematists differ as to whether the tribe Thalassinidea, to which *Upogebia* belongs, should be placed with the Anomura or the Macrura. In 1960, Waterman and Chace included this tribe with the Macrura. On the other hand, in 1961, Green classified the Thalassinidea among the Anomura.

The optic ganglia and sinus glands of the mole crab, *Emerita talpoida,* and of *Upogebia* are closely associated with the supraesophageal ganglia instead of occurring in the eyestalks as is the case in most decapods studied. According to Hanström (1937) the retinal structures and their associated nerve tracts are the only major components of the nervous system present in the eyestalks of these two species. In contrast, the eyestalk of the anomuran, *Pagarus pollicaris,* a hermit crab, contains the usual ganglia, such as the medulla terminalis, and the sinus gland, in addition to the visual structures (Hanström, 1937).

Perkins and Kropp (1932) noted that eyestalks of *Pagurus longicarpus* blanched the shrimp, *Crangon boreas,* which is not an anomuran. In contrast, no response was observed when extracts of eyestalks from *Upogebia* and *Emerita* were injected into the fiddler crab, *Uca,* by Carlson (1936) or into the prawn, *Palaemonetes,* by Hanström (1937). But Hanström did find that eyestalks of *Pagurus pollicaris* blanched eyestalkless *Palaemonetes.* Head extracts of *Upogebia,* on the other hand, darkened eyestalkless *Uca* (Carlson, 1936) and blanched eyestalkless *Palaemonetes* (Hanström, 1937). However, the question remained whether the effect of the head extracts was due to the sinus glands, the supraesophageal ganglia, or both structures, until Sandeen and Baldwin (1962) assayed glands and supraesophageal ganglia of *Upogebia* on *Uca.* Both organs yielded extracts that caused even more melanin dispersion than did extracts of the same tissues from *Uca* when assayed on *Uca.*

In the meantime, Brown and Scudamore (1940) had postulated the presence of

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at least two chromatophorotropins in the sinus gland of *Pagurus pollicaris*. Brown and Saigh (1946) found two antagonistic chromatophorotropins in central nervous organs of *Upogebia affinis, Emerita talpoida, Pagurus pollicaris,* and *P. longicarpus*. Because the assays were performed on *Crangon,* the antagonists in the central nervous organs were termed *Crangon* body-lightening hormone (CBLH) and *Crangon* darkening hormone (CDH).

Through the efforts of several investigators working in the early 1950's (*e.g.,* Passano, 1951; Bliss and Welsh, 1952), the sinus gland was shown to be a storage and release organ, *i.e.,* a neurohemal organ, rather than an actual site of hormone production. Chromatophorotropins appear to be products of neurosecretory cells, transported by axoplasmic flow from the site of formation to neurohemal organs. Miyawaki (1960) is the only investigator who described the cytology of neurosecretory cells in anomurans. He found three types of such cells in the central nervous organs of the crabs *Eupagurus ochotensis* and *Paralithodes brevipes*.

The literature concerning (a) the morphology of neuroendocrine organs and (b) the physiology of chromatophores in typical Macrura is very extensive. In such forms the sinus gland typically resides in the eyestalk (Hanström, 1937). The chromatophore system of Macrura is highly evolved, pigment-concentrating and pigment-dispersing principles having been demonstrated (Fingerman and Aoto, 1962).

The general object of this investigation was to learn the origins and actions of chromatophorotropins in *Upogebia*. The specific aims were to determine (1) the responses of *Upogebia* to extracts of its own eyestalks, sinus glands, and supraesophageal ganglia, and (2) the distribution of neurosecretory cells in the head of *Upogebia*.

MATERIALS AND METHODS

We are indebted to the personnel from the Supply Department of the Marine Biological Laboratory at Woods Hole, Massachusetts, and to Dr. Muriel I. Sandeen of the Duke Marine Laboratory at Beaufort, North Carolina, for furnishing specimens of *Upogebia affinis*. Specimens used in the bioassays were maintained in the laboratory at Woods Hole in aquaria supplied with constantly flowing sea water.

Red chromatophores on the dorsal surface of the telson and uropods were staged according to the system of Hogben and Slome (1931). Stage 1 represents maximal concentration of the pigment, stage 5 maximal dispersion, and stages 2, 3, and 4 the intermediate conditions. Student's t test was used in the statistical analysis of the data.

Tissue extracts were prepared by grinding the appropriate number of organs in sea water. The concentration was one-third of the organ complement from one mud shrimp per dose, 0.05 ml. Therefore, each dose contained either both eyestalks, both sinus glands, or the supraesophageal ganglia from one mud shrimp.

Paraffin sections of the eyestalks, sinus glands, and supraesophageal ganglia were prepared in the usual fashion. These structures were fixed in (1) Bouin's solution or (2) Helly's solution. Sections 8 and 10 μ thick were stained with (1) Mallory's trichrome, (2) Heidenhain's azan, (3) Gomori's chrome alum hematoxylin-phloxin, or (4) aldehyde fuchsin.

Observations and Results

Responses of erythrophores in Upogebia to tissue extracts

The aim of this experiment was to observe the effects of eyestalks, sinus glands, and supraesophageal ganglia from *Upogebia* on eyestalkless specimens of *Upogebia*. The results are presented in Figure 1 where the results from three experiments (3, 3, and 4 test animals, respectively) are averaged.

Aside from the responses to the extracts, inspection of Figure 1 reveals that the red pigment of eyestalkless *Upogebia* was maximally dispersed before injection of the extracts and sea water. Injection of the tissue extracts caused statistically significant degrees of pigment concentration, $p < 0.001$ for each extract, despite the fact that control injections of sea water evoked some pigment concentration. The chromatophore indexes measured 15 minutes after injection of extracts were used for the statistical calculation. Responses of the chromatophores were highest to extracts of supraesophageal ganglia and least to eyestalk extracts.

Neurosecretory cells in Upogebia

In view of the observation (Fig. 1) that extracts of the supraesophageal ganglia, sinus glands, and eyestalks of *Upogebia* caused a significant concentration of the red pigment in this organism, investigation of these structures for signs of neuro-

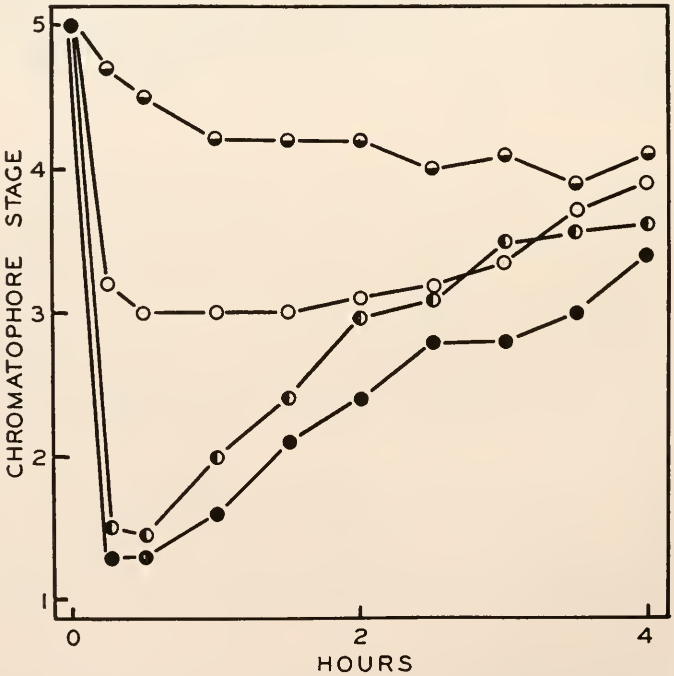

Figure 1. Relationships between chromatophore stage and time following injection of tissue extracts into eyestalkless *Upogebia*. Circles, eyestalks; dots, supraesophageal ganglia; circles half filled on left, sinus gland; circles half filled on bottom, control.

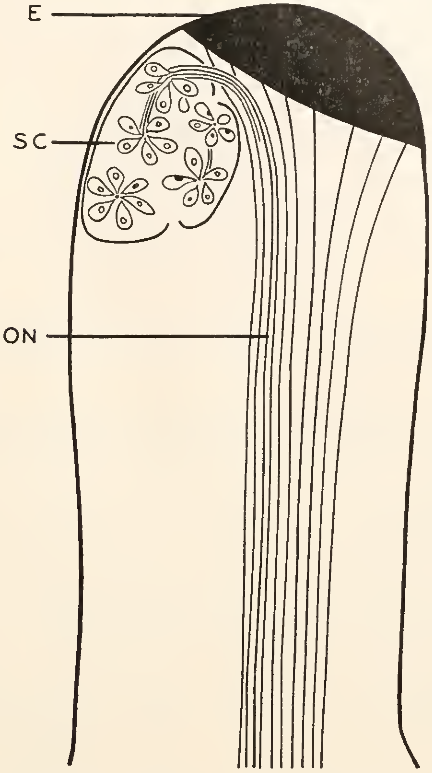

FIGURE 2. Diagram of the eyestalk of *Upogebia.* E, eye; ON, optic nerve; SC, secretory cells.

secretory activity seemed appropriate. Examination of sectional eyestalks revealed a group of 500–700 ovate cells whose cytology and staining properties are typical of neurosecretory cells. This cluster of cells lies in the dorsal half of the distal third of the eyestalk, partially surrounding the optic nerve, and is enclosed by a connective tissue sheath (Fig. 2). The cells are fairly uniform in size, averaging 14 μ wide and 19 μ long, with an axon emerging from one end. The nucleus, which has a conspicuous nucleolus, is centrally located in some of the cells, eccentrically in others. The cytoplasmic granules appear to be neurosecretory products. For example, they stain pink with Heidenhain's azan and are positive to aldehyde fuchsin. In addition to the granules, some of the cells have cytoplasmic vacuoles. The cells are arranged in clusters resembling rosettes (Fig. 3). Because of the unique architecture of this group of cells, the structure will be referred to as the Rosette Body. Blood sinuses were noted in both the central and peripheral portions of the Rosette Body. Pores in the connective tissue sheath afford the blood ready access to these sinuses.

Axons of the optic nerves contained granules that appeared to be neurosecretory products. These granules probably originated in the Rosette Body, inasmuch as the Rosette Body was the only structure observed in the eyestalk that could be the

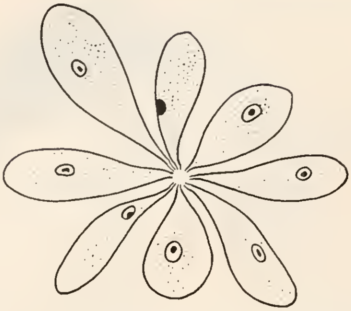

FIGURE 3. Detailed diagram of the cell arrangement in a portion of the Rosette Body.

source of the secretory granules in the optic nerve. The axons from the Rosette Body appear to unite with the optic nerve just proximal to the basement membrane of the eye. Axons of the optic nerve could not be followed after they entered the supraesophageal ganglia.

The general morphology of the sinus glands and supraesophageal ganglia conforms to the description and photograph presented by Hanström (1937). Inspection of histological sections of the supraesophageal ganglia revealed the presence of three distinct types of cells that had neurosecretory staining properties. The largest type has a cell body averaging 24 μ wide and 40 μ long and is restricted to the medullae terminales. The left and right medullae terminales are fused in the midline of the supraesophageal ganglia, as described by Hanström (1937). About six cells of this type occur. They have a round nucleus in the center of the cell body. A conspicuous nucleolus lies near the nuclear membrane. Near the periphery of the cytoplasm occur several vacuoles. The second type, similar in size and shape to the cells that compose the Rosette Body, possesses a large nucleus with a conspicuous nucleolus. Some of these cells are vacuolated. The third type has a round, non-vacuolated cell body 9–11 μ in diameter. This cell is small as far as the usual neurosecretory cell is concerned. Only in the medullae terminales were all three types observed. The sinus gland consisted of nerve endings alone, no cell body having been observed. Of all the ganglia, the medullae terminales had the highest proportion of neurosecretory cells to ganglionic cells.

DISCUSSION

The eyestalks, supraesophageal ganglia, and sinus glands of *Upogebia* possess a principle that concentrates the red pigment in this animal (Fig. 1). As mentioned above, Carlson (1936) and Sandeen and Baldwin (1962) working with *Uca,* and Hanström (1937) working with *Palaemonetes* as assay animals, found that eyestalk extracts of *Upogebia* were ineffective. However, eyestalk extracts of *Upogebia* were effective on *Upogebia* (Fig. 1). The species differences are a possible explanation of the lack of response observed by the other investigators. The greater potency of extracts of supraesophageal ganglia from *Upogebia* compared with sinus gland extracts from this animal (Fig. 1) was also noted by Sandeen and Baldwin (1962) who assayed these extracts on *Uca*.

In *Upogebia* the medulla terminalis X-organ lies in the supraesophageal ganglia. The group of previously undescribed cells that compose the Rosette Body in the eyestalk of *Upogebia* (Figs. 2 and 3) may be homologous with the sensory pore X-organ described by Hanström (1939) in the eyestalk of many crustaceans, *e.g., Palaemon, Crangon,* and *Homarus.* He believed that this organ represents transformed sensory cells of a rudimentary eye papilla or sensory pore. Concentration of the erythrophores in *Upogebia* following injection of the eyestalk extract was presumably due to the secretory product of the Rosette Body. This secretory material may normally be transported by axoplasmic flow into the supraesophageal ganglia and from there to the sinus glands for storage.

Nishida and Miyawaki (1954) described a holocrine gland in the eyestalk of two species of the anomuran *Paralithodes.* In *Paralithodes* the medulla externa, medulla interna, and medulla terminalis occur in the eyestalk but no sinus gland was observed. It may lie on the surface of the supraesophageal ganglia as in *Upogebia.* However, the structure described by Nishida and Miyawaki is not the Rosette Body of *Upogebia.* The present report constitutes the first description of the occurrence of an organ that presumably contains neurosecretory cells in the eyestalks of a crustacean whose optic ganglia do not lie in the eyestalk. Furthermore, the arrangement of the cells is unique for a neurosecretory organ.

As mentioned above, Miyawaki (1962) described three types of neurosecretory cells in the anomurans *Eupagurus ochotensis* and *Paralithodes brevipes.* The widths of the cell bodies were 100–130 μ, 30–60 μ, and 10 μ. In *Upogebia* three sizes of neurosecretory cells occur also. However, the largest type described by Miyawaki is much bigger than any that occurs in *Upogebia.*

The problem of whether *Upogebia* is an anomuran or a macruran should be resolved. The chromatophore system of *Upogebia* does not allow us to decide between these alternatives because so little is known about chromatophore responses of anomurans in general that speculation would be meaningless. On the other hand, the neurosecretory system does offer a clue. The secondary return of the medullae terminales from the eyestalks to the head is typical of anomurans with reduced eyes rather than of macrurans (Hanström, 1948).

SUMMARY AND CONCLUSIONS

1. The eyestalks, sinus glands, and supraesophageal ganglia of the mud shrimp, *Upogebia affinis,* contain a principle that concentrates the pigment in its red chromatophores.

2. A previously undescribed group of cells, with tinctorial properties characteristic of neurosecretory cells, occurs in the eyestalks.

3. Because the arrangement of the cells in this structure is unique among neurosecretory organs, it is proposed that this structure be called the Rosette Body. The secretory product of these cells is probably conveyed through the optic nerve by axoplasmic flow to the supraesophageal ganglia and from there to the sinus glands.

4. The Rosette Body may be homologous with the sensory pore X-organ of higher crustaceans.

5. Three types of cells with tinctorial properties characteristic of neurosecretory cells occur in the supraesophageal ganglia.

6. These observations were discussed in relation to the findings of other investigators.

LITERATURE CITED

Bliss, D. E., and J. H. Welsh, 1952. The neurosecretory system of brachyuran Crustacea. *Biol. Bull.,* **103**: 157–169.

Brown, F. A., Jr., and L. M. Saigh, 1946. The comparative distribution of two chromatophorotropic hormones (CDH and CBLH) in crustacean nervous systems. *Biol. Bull.,* **91**: 170–180.

Brown, F. A., Jr., and H. H. Scudamore, 1940. Differentiation of two principles from the crustacean sinus gland. *J. Cell. Comp. Physiol.,* **15**: 103–119.

Carlson, S. P., 1936. Color changes in Brachyura crustaceans, especially in *Uca pugilator. Kungl. fysiogr. Sällsk. Lund Forhandl.,* **6**: 63–80.

Fingerman, M., and T. Aoto, 1962. Hormonal regulation of pigmentary effectors in crustaceans. *Gen. and Comp. Endocrinol. Suppl.,* **1**: 81–93.

Green, J., 1961. A Biology of Crustacea. Quadrangle Books Inc., Chicago.

Hanström, B., 1937. Die Sinusdrüse und der hormonal bedingte Farbwechsel der Crustaceen. *Kungl. Svenska Vetenskap. Handl.,* **16**: No. 3, 1–99.

Hanström, B., 1939. Hormones in Invertebrates. Oxford University Press, Oxford.

Hanström, B., 1948. The brain, the sense organs, and the incretory organs of the head in the Crustacea Malacostraca. *Bull. Biol. France et Belg. Suppl.,* **33**: 98–126.

Hogben, L. T., and D. Slome, 1931. The pigmentary effector system. VI. The dual character of endocrine co-ordination in amphibian colour change. *Proc. Roy. Soc. London, Ser. B,* **108**: 10–53.

Miyawaki, M., 1960. On the neurosecretory cells of some decapod Crustacea. *Kumamoto J. Sci., Ser. B,* **5**: 1–20.

Nishida, H., and M. Miyawaki, 1954. Occurrence of a remarkable glandular body in the eyestalk of *Paralithodes camtschatica* and *P. brevipes. Zool. Magazine, Japan,* **63**: 274–277.

Passano, L. M., 1951. The x organ-sinus gland neurosecretory system in crabs. *Anat. Rec.,* **111**: 86–87.

Perkins, E. B., and B. Kropp, 1932. The occurrence of the humoral chromatophore activator among marine crustaceans and its effect upon the chromatophores of crustaceans, fishes, and Amphibia. *The Mount Desert Island Biol. Lab. Annual Report,* pp. 24–26.

Sandeen, M. I., and M. F. Baldwin, 1962. The comparative distribution of fiddler crab chromatophorotropins in the burrowing shrimp, *Upogebia affinis,* and the isopod, *Ligia exotica. Amer. Zool.,* **2**: 554.

Waterman, T. H., and F. A. Chace, Jr., 1960. General crustacean biology. *In:* The Physiology of Crustacea. Vol. I, Metabolism and Growth, T. H. Waterman, ed. Academic Press, New York.

THE BIOLOGY OF ASCIDIA NIGRA (SAVIGNY). II. THE DEVELOPMENT AND SURVIVAL OF YOUNG ASCIDIANS[1]

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In the first paper in this series (Goodbody, 1962) a description was given of the survival and mortality of ascidians after they first appeared as black animals visible to the naked eye. Because of the technique used for studying those populations it was believed that most of them were about four weeks old when first recorded, and no information was available on the survival of animals of younger age. The work outlined in the present paper was designed to study two things: first, to study the development of ascidians from fertilization of the egg to the appearance of black pigment in the functional animal, with a view to producing a time scale for the different phases of development. Second, to study the survival of young animals from first settlement to the appearance of black pigment, and to determine whether there was any relationship between mortality rate and the different stages of development. While this has been achieved in some measure, it will become apparent that a far more fundamental problem emerges from it which will require further study. This is the relation of changes in the biotic environment to patterns of survival.

METHODS

Populations of young ascidians were obtained either artificially in the laboratory or through natural settlement on glass slides in the sea. Laboratory-reared populations were obtained through artificial fertilization of ascidian eggs: these hatched in 10 hours and the larvae were then pipetted gently into a perspex (lucite) settling box. This box, approximately 4 inches square and 2½ inches in height, has walls of black perspex, a base of clear transparent perspex and an open top. The box is designed so that four standard microscope slides can be fitted, standing up edge to edge along each wall, in such a way that the inside of the box is lined with glass. One face of each slide is gently ground with carborundum to provide a roughened surface for settlement; by means of small clips at the top the slides can be secured with this roughened surface facing the inside of the box. With the slides in place and the box filled with water, the larvae were pipetted into it and the box then placed on a stand in an aquarium tank so that the flow of water outside the box kept the water inside cool. The clear base to the box allows light to come into it from below as well as from above: the larvae, which tend to settle on dark surfaces, were thus induced to settle on the glass slides backed by the dark wall of the box. The box was left thus for about six hours until such time as the larvae had settled.

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[1] Contribution No. 313 from the Bermuda Biological Laboratory.

Attached larvae can be seen readily with the naked eye when a slide is withdrawn from the water.

When settlement was complete the slides were transferred to an open rack in the aquarium and left there overnight to ensure proper attachment before they were further disturbed. The following day each slide was transferred in turn to a perspex dish mounted on the moving stage of a binocular microscope. This dish is designed to contain sufficient water to cover the slides adequately during inspection, and has two projecting ledges on the inside base to enable each slide to be positioned in exactly the same place at successive inspections. In this way, and utilizing the graduated scales of the moving stage, it was possible to plot accurately the position of every young ascidian on each slide. When this was complete the slides were mounted edge to edge in a perspex frame backed by black perspex. (For further details of these frames, see Goodbody, 1961.) The black backing to the slides in the frames served two functions: it prevented any settlement of other organisms on the back of the slides which hence continued to fit properly into the microscope dish, and by providing a dark background it encouraged settlement of other organisms on the surface occupied by the ascidians, thus providing a natural community in which the ascidians could develop. These frames containing the slides were then suspended in the sea from floating rafts as described earlier (Goodbody, 1962). At intervals throughout the following six weeks the frames were carefully removed to the laboratory in a bucket of water; the slides were removed to a rack in the aquarium and examined in turn to determine how many of the original population still survived. To avoid any possible contamination while in the laboratory, special containers were reserved and used only for the transport and handling of these frames.

Naturally-occurring populations were also obtained on microscope slides. Clean slides similar to those described above were fitted into frames and placed in the sea, suspended from a raft. At intervals the slides were removed and inspected for newly settled larvae of *Ascidia nigra,* which can be identified at this stage (*cf.* Goodbody, 1961). Although newly settled larvae were repeatedly observed in small numbers, only two populations of sufficient size, and each comprising a natural cohort of animals derived from a single settlement, have been recorded and followed through their subsequent history. These two, however, provide a valuable supplement to the data obtained from the artificially settled populations.

The data on the developmental stages of the young ascidian were first worked out at Bermuda and have been confirmed and added to from observation and measurement of animals within these populations. Measurements were made with the aid of a micrometer eye-piece. All the data on survival were obtained at Port Royal, Jamaica.

The Sequence of Early Development

The sequence of events leading from the fertilization of the egg to the appearance of a black ascidian, visible to the naked eye, can be divided into stages as follows: embryonic development, the free swimming larva, settlement and metamorphosis, the functional protoascidian with two protostigmata on each side, the six protostigmata stage, twelve stigmata, six rows of eight stigmata, twelve rows of stigmata and developing red pigmentation, and finally the opaque black ascidian. This whole sequence of events takes 19 days from the fertilization of the egg, at temperatures in the vicinity of 27° C. Illustrations of comparable stages in young ascidians are to be found in the papers of Berrill (1935, 1947).

The unfertilized egg measures about 160 μ in diameter; fertilization is external and the first cleavage (under laboratory conditions) follows in about 30 minutes. Subsequent cleavages follow in rapid succession, and the free swimming larva hatches 9 to 10 hours after fertilization. Grave (1935) gives a developmental time of 8 hours for this species at temperatures between 27 and 29° C. The same author's (1925) developmental time of 6 hours and 38 minutes was recorded at the exceptionally high temperature of 33° C. The total length of the larva is about 825 μ, of which 200 μ comprise the length of the body and the remaining 625 μ the tail. Larval behavior has not been studied in detail but, in common with most other ascidian species, the larvae of *A. nigra* are at first positively, and later negatively, phototropic. They are probably actively attracted towards iron, as submerged iron structures, which are not painted, often have dense growths of this species attached to them. Grave and Nicoll (1940) showed that iron accelerates metamorphosis in *A. nigra*.

Settlement of larvae under laboratory conditions usually occurs within 3 to 6 hours of hatching but has sometimes been delayed for as long as 12 hours. Grave and Nicoll (1940) give times ranging from 7½ to 30 hours at Tortugas, Florida, according to the time of year. In field conditions an extended larval life may be more common, particularly if the larvae take a long time to find a suitable substratum on which to settle. When settlement is complete the tissues of the tail, except for the surrounding test, are resorbed into the body of the animal and accumulate there as a ball of nutrient reserves which nourish the growing animal until the gut is functional; these reserves have usually completely disappeared within 24 hours of the commencement of feeding by the functional animal. In the first stages of metamorphosis, when the tail has just been resorbed, the animal measures about 200 μ in length but this increases to 250 μ by the time the functional protoascidian commences feeding; this stage is reached about 45 hours after settlement of the larva.

The sequence of events from settlement of the larva is summarized in Table I, but it must be emphasized that there is some variation in the times and sizes given. A new stage of differentiation will only occur when the animal has reached a definite size: under conditions of poor food supply, growth may be retarded and hence the time scale will be correspondingly lengthened.

The functional protoascidian has two protostigmata on each side of the branchial sac, a single branchial aperture, paired peribranchial apertures, and a functional gut with oesophagus, stomach and intestine. Simultaneously with the commencement of feeding the first renal vesicle can be seen, close to the oesophagus. During the subsequent two days there is little new differentiation but the animal grows from about 250 μ to 550 μ and at the same time the nutrient reserves from the tail tissues completely disappears. Between the fifth and ninth days, and while the animal continues to grow, the original two protostigmata divide in such a way that there is formed, first a single row of six protostigmata, and then six rows of definitive stigmata on each side. There is considerable variation in the sequence

TABLE I

Time scale for the development of young Ascidia nigra from settlement to the appearance of black pigment

Time from settlement of larva	State of development	Approximate length (μ)
24 hours	Branchial siphon, 2 protostigmata, gut and heart just visible. Heart just commencing to beat. Large food reserves.	200
45–48 hours	Functional protoascidian with 2 pairs of protostigmata, food in gut, 1 renal vesicle. Food reserve small. Paired peribranchial apertures.	200–300
48–96 hours	Growth of the protoascidian and complete disappearance of food reserve. No new differentiation.	250–625
5–6 days	Differentiation of 6 protostigmata on each side. Branchial siphon develops lobes and red pigment spots between them. At least three renal vesicles.	500–700
7–8 days	Six protostigmata divide into 6 pairs of stigmata. Peribranchial apertures move toward mid-line and fuse. Four branchial tentacles visible. At least 9 renal vesicles.	700–1150
8–12 days	Six rows of stigmata completed. Peribranchial siphon becomes lobulated and develops red pigment spots.	1125–1875
13–15 days	Twelve rows of stigmata completed. Red pigmentation begins to develop.	1800–2400
19 days	Opaque black all over.	2750

of events occurring, and sometimes the protostigmata in the center of the row of six will have divided into three or more parts before the outer ones have completed their first division. On the fifth or sixth day the branchial siphon becomes lobed on its margins and red pigment spots develop between the adjacent lobes. The two peribranchial apertures begin to move towards the dorsal mid-line on about the seventh day, and by the eighth they have fused to form a single atrial siphon. Grave (1925) recorded fusion of the peribranchial apertures between the seventh and ninth days.

After the ninth day there appears to be a slight pause as further growth occurs up to a maximum of nearly 1900 μ, before the six rows of stigmata again divide to give twelve rows of stigmata on either side. It is at this stage, between the thirteenth and fifteenth days for normally growing animals, that the first signs of pigmentation appear. The pigment arises as a deep red coloration all over the animal which gets progressively darker until the eighteenth day from settlement, and at a size of about 2750 μ, the animal is so black that no further details of internal development can be observed.

Developmental times for other species of simple ascidian have been given by Berrill (1935) and Millar (1951, 1954). For *Ascidiella aspersa* (Müller) and

Ciona intestinalis (Linnaeus) at 16° C., Berrill records hatching after 24 hours; commencement of heart beat at 150 hours; appearance of functional protostigmata at 260 hours, and six rows of eight stigmata at 6 to 8 weeks. In comparison with these data for *Ciona* and *Ascidiella,* and allowing for a difference of 11° C. in the environmental temperature, the development of *A. nigra* seems extremely rapid. All the figures given are consistent with its development occurring six times faster than that of the other two species. Millar records the functional protostigmata stage as being reached in 10 days in both *Pyura squamulosa* (Alder) and *P. microcosmus* (Savigny) at temperatures in the vicinity of 20° C., which again is about six times as long as *A. nigra* takes to reach the same stage at 27° C.

SURVIVAL

As indicated earlier, survival has been studied in two types of poulation. In the first place there are data for seven different populations derived from artificial fertilization and subsequent settlement in the laboratory. As soon as settlement was complete and metamorphosis had begun, these populations were transferred to frames in the sea. Second, there are data from two other populations which settled naturally on microscope slides placed in frames in the sea for that purpose. The environmental histories of these different types of population are different, and comparison of the two must be made with caution. The populations derived from artificial fertilizations were allowed to settle on completely clean slides on which there were no competitors at the times of settlement; they were, in fact, the first colonizers of these slides and other colonizers apeared only after the slides had been placed in the sea, usually about 12 hours after settlement had taken place. This is an unnatural situation. The other populations appeared on the slides only when a sessile community had already begun to develop: in one of them, Group H, the ascidian larvae did not settle in the community until it was 16 days old; in the other (I) they settled after the slides had been in the sea for only two days.

The data on these nine populations are presented in Tables II and III. Table II shows the total number of animals in each population, together with the date of settlement and depth at which it was reared. In Table III the survival of each population is presented in terms of initial cohorts of 1000 animals each. Special note should be taken of population H, which settled naturally but was not observed until the third day after settlement. At this time there were 218 animals present.

TABLE II

Total number of animals, date of settlement and depth at which populations were reared. Note that the number of animals in population H is a re-calculated figure from 218 animals observed on third day (*see text*)

Population	A	B	C	D	E	F	G	H	I
Total no. of animals	80	488	173	148	72	105	79	271	34
Date of settlement	23.6.61	30.6.61	7.8.61	7.8.61	11.8.61	11.8.61	31.1.59	24.9.61	23.10.61
Depth in feet	4	4	4	7	4	7	7	7	7
Artificial or natural settlement	A	A	A	A	A	A	A	N	N

TABLE III

Survival rate (1x) in 9 populations of young Ascidia nigra, *presented as cohorts of 1000 animals each. For further data on these populations, see Table II.*

No. of days since settlement	Population								
	A	B	C	D	E	F	G	H	I
0	1000	1000	1000	1000	1000	1000	1000	1000	1000
1	950	—	960	919	—	—	—	—	—
2	862	846	873	865	—	—	—	—	—
3	525	814	815	831	639	638	—	803	941
4	437	773	—	—	583	571	342	—	—
5	362	748	—	—	—	—	—	635	—
6	—	619	—	514	333	495	—	—	—
7	—	518	486	—	208	448	—	—	—
8	275	—	—	—	181	400	—	565	588
9	—	—	—	365	—	—	215	—	—
10	262	434	231	—	111	286	—	458	—
11	—	—	—	223	—	—	—	—	500
12	250	—	—	—	55	267	—	—	—
13	—	348	145	—	—	—	—	362	—
14	238	—	—	162	42	210	—	—	—
15	—	—	87	—	—	—	—	—	353
16	—	299	—	135	—	—	76	273	—
17	—	—	58	—	42	162	—	—	—
18	238	279	—	122	—	—	—	—	—
19	—	—	—	—	14	76	—	177	—
20	—	—	17	—	—	—	—	—	—
21	225	240	—	—	14	67	—	—	176
22	—	—	17	81	—	—	—	—	—
23	225	200	—	—	—	—	51	—	—
24	—	—	0	61	—	—	—	48	—
25	—	191		—	—	—	—	—	118
26	200	—		—	0	57	—	—	—
27	—	—		—		—	—	30	—
28	187	154		—		—	—	—	58
29	—	—		61		—	—	—	—
30	—	—		—		—	38	—	—
31	—	—		—		—		—	—
32	—	129		—		—		—	—
33	175	—		—		48		—	—
34	—	—		—		—		11	—
35	—	127		—		—		—	—
36	—	—		—		—		—	—
37	—	—		41		—		—	—
38	—	—		—		—		—	—
39	—	119		—		—		—	—
40	—			—		—		—	—
41	—			—		—		—	—
42	—			—		29		—	30
43	175			—				—	
44				—				—	
45				20				3.7	

The figure of 271 given in Table II is a re-calculated value of the probable size of the original population when it settled. This has been calculated from the mean of the known survival in populations D, F and I on the third day; these populations are chosen because they were reared at the same level as H, and data are available for the third day of life. The re-calculation introduces a possible error in the data, but without it we cannot compare this interesting natural population with those which were artificially reared. The extent of the error is not large and is confined to the position of the first point on the survival curve and the percentage of survivors remaining at the terminal point; the intervening curve would be displaced but not altered in form. The percentage of survivors at the end might be depressed as low as 0.24% or elevated to 0.45% as against 0.37% resulting from the re-calculation. These figures are based on the assumption that the highest mortality in the first three days might be equivalent to that shown by population A (47.5%), which would result in 0.24% of survivors at the terminal point, and the extreme possibility that there had been no mortality in the first three days when there would be 0.45% of survivors at the end; both alternatives are improbable.

The data are presented graphically in Figures 1 to 4. It will be apparent from these figures that there is not one clear-cut type, but several groups, of survival patterns. Populations A and B form one grouping, with 17.5% and 12% survival after 40 days; populations D, F, G and I form a second grouping, with between 2% and 3% survival after the same period, and populations C and E form a third group, all of which had been lost by the twenty-fifth day. Population H, the large, naturally settled population, followed the pattern of D, F, G and I at first but diverged markedly after about 20 days; it is discussed further below.

These different survival patterns can, to some extent, be related to differences in the environment under which the populations were reared. If we consider populations A, B, C and E at first, we find that all four populations, derived from artificial fertilizations, were reared in the sea at a depth of four feet below the surface; since they were suspended from a floating raft this depth remained constant, irrespective of tidal movements. Populations A and B were derived from fertilizations at the end of June, C and E were from fertilizations in early August. In early August and through September of 1961, when these experiments were carried out, there was a dense settlement of algal spores and subsequent growth of filamentous algae on all frames at the four-foot level. The frames with populations A, B, C and E were all equally affected by algal growth. The continued survival of populations A and B and the total loss of populations C and E under these conditions may be explained by the fact that the survivors in A and B were, by this time, quite large animals, while the survivors in C and E were still in the early stages of development and probably more susceptible to smothering by algae. We do not have sufficient data on other environmental factors at these times to be certain that it was the algae and not something else which caused the rapid decline of C and E. However, we may now compare these two populations with populations D and F, also derived from artificial fertilizations in early August. D and F were reared at 7 feet below the surface, left 3% of survivors after 6 weeks and did not have the same dense growth of algae on the frames as did C and E. This, then, strengthens the conclusion that the dense algal growth may have been responsible for the decline in populations C and E. Populations C and D were derived from the same

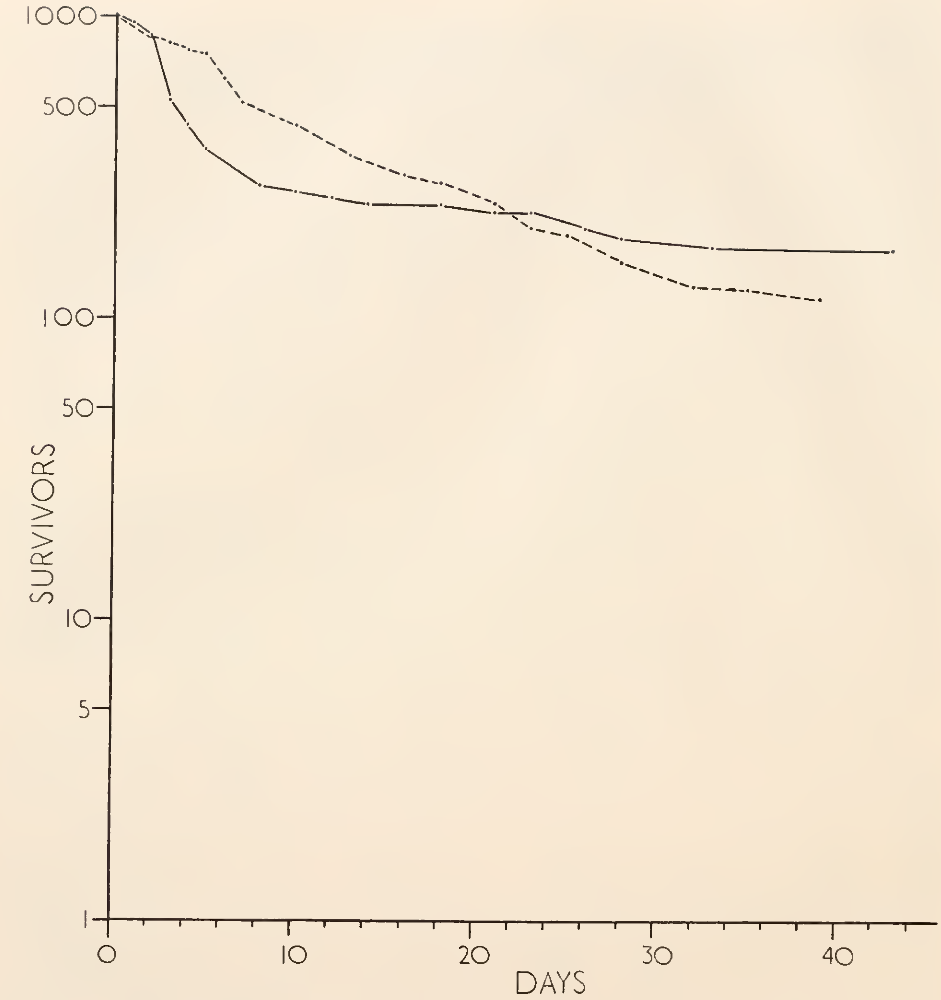

Figure 1. Survival in two populations of young ascidians, A (————) and B (— — — —), settled in June, 1961, and reared four feet below the sea surface.

fertilization and were placed in the water on August 7th, the one three feet below the other in the water; the deeper one, D, with little algal growth, had a higher rate of survival than the upper algal-covered population C. Similarly, populations E and F were derived from a single fertilization on August 11th; F was kept three feet below E and had more survivors.

The high level of survival in populations A and B in comparison with D and F is also of interest but not readily explained. A and B were derived from fertilizations in late June and were maintained four feet from the surface; D and F were derived from fertilizations in early August and were maintained 7 feet from the surface. The difference in survival between the two sets of populations may be due to an environmental factor, possibly a biotic factor concerned with competition from other sessile organisms. Similar sorts of communities developed with each population dominated by cirripedes, serpulids and colonial ascidians. The data on the associated fauna and flora are necessarily rather superficial but there is nothing in the record to suggest that one community differed much from the other. Attention should be drawn to the fact that June is one of the months when breeding in *A. nigra* is normally at a minimum (Goodbody, 1961), suggesting that this is a poor time of year for breeding and subsequent survival. August, on the other hand, is at the beginning of the autumnal rise in breeding activity of this species, suggesting that environmental conditions for breeding and survival of the young are improving.

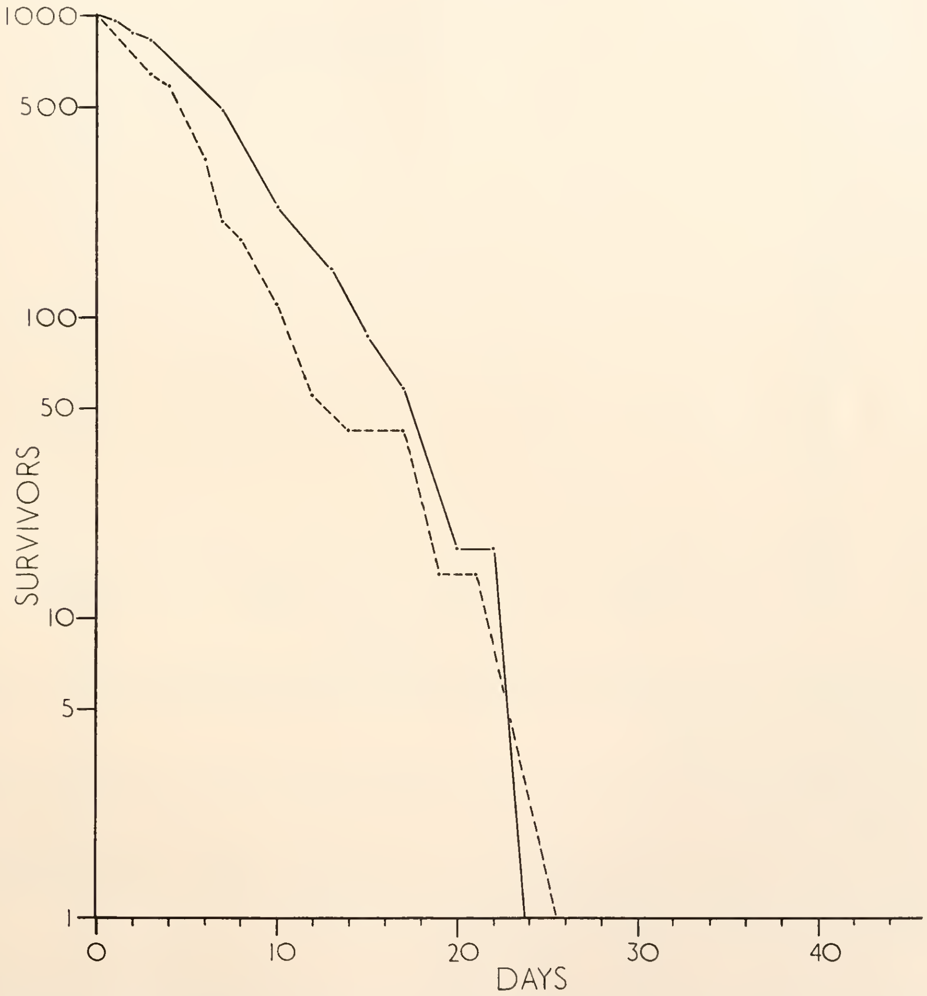

FIGURE 2. Survival in two populations of young ascidians, C (————) and E (— — — —), settled in August, 1961, and reared four feet below the sea surface.

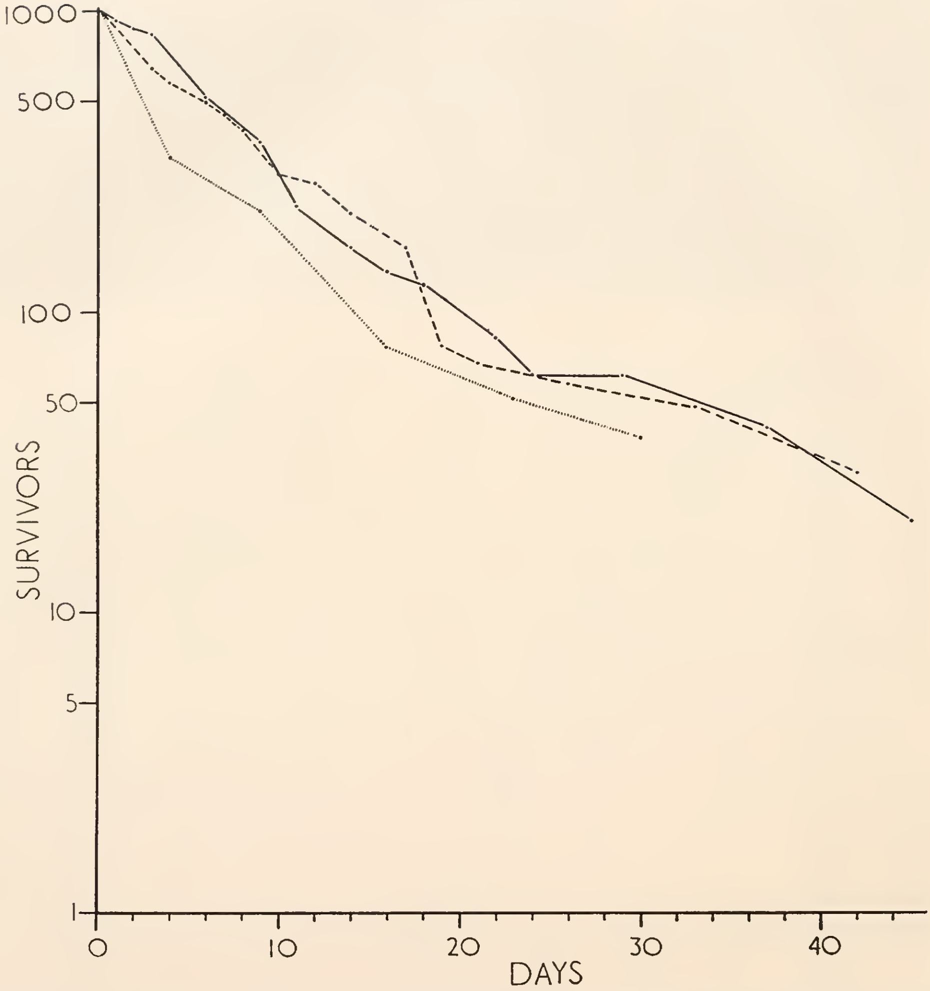

FIGURE 3. Survival in three populations of young ascidians reared 7 feet below the sea surface. D (————) and F (— — — —) were settled in August, 1961. G (..........) was settled in January, 1959.

It is surprising, therefore, to find that the June population shows such a high level of survival.

There remain three other populations to be discussed. G was a population set up in January, 1959, and maintained 7 feet below the surface. It was followed for only 30 days and until then showed a survival pattern closely similar to those populations settled in August and maintained at 7 feet.

Finally, there are data on two populations derived from fertilizations in the sea, which settled naturally on slides at 7 feet. H was a large population settling on slides which already had two weeks' growth of barnacles and other sessile forms. I settled two days after slides had been placed in the water and is therefore more nearly comparable to the artificial settlements; it is, however, a small population compared to any of the others.

Population I shows a survival curve closely approximating that of the artificial populations D, F and G, reared at 7 feet, and like them had 3% survival at the end of 6 weeks. However, it must be emphasized that this represents no more than one animal surviving. Population H follows the pattern of D, F, G and I until about the twentieth day, when the curve falls sharply, and the final survival at the forty-fifth day is only 0.37%, but this also represents only one surviving animal. Nevertheless, the curve merits some attention, as there is a possible explanation for the divergence between it and the others. The animals in this population settled in a

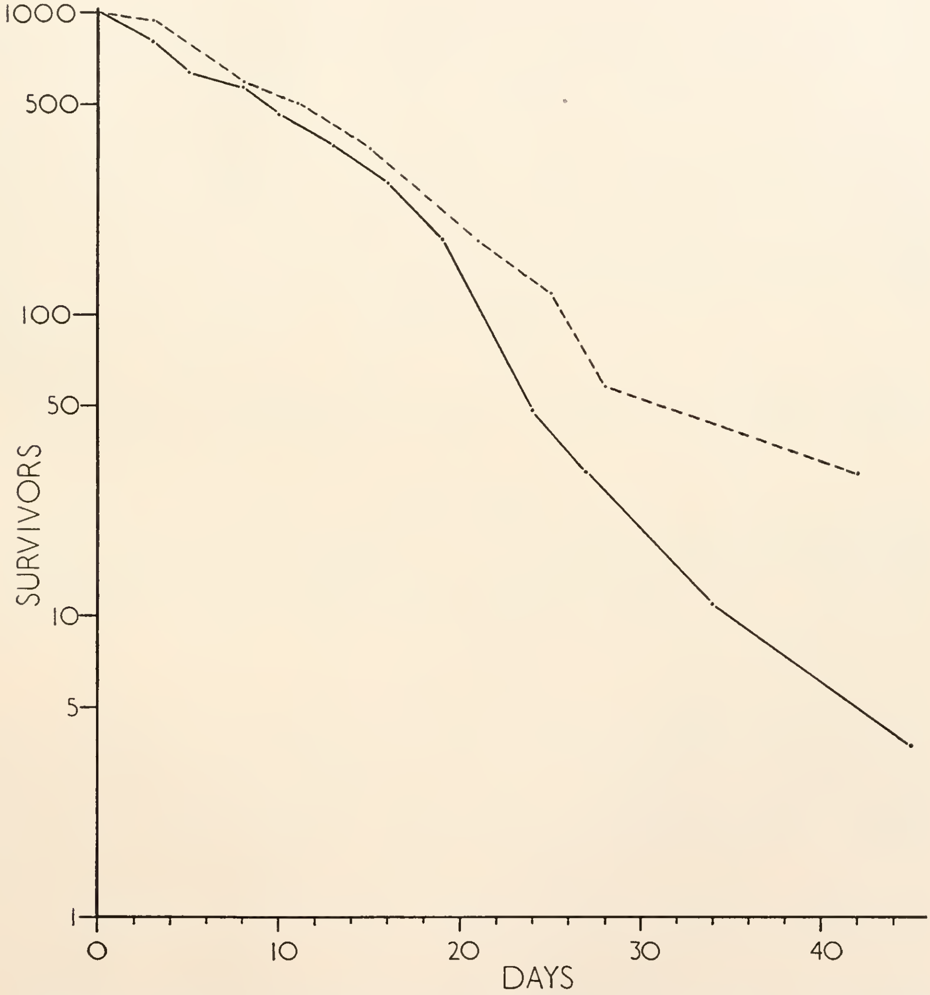

FIGURE 4. Survival in two naturally settled populations of young ascidians, 7 feet below the sea surface. H (————) settled in September, 1961, and I (— — — —) settled in October, 1961. The data for H have been re-calculated to obtain the first point on the curve (see text).

population which was already 16 days old, whereas those in population I settled in one which was two days old, and D, F and G were first colonizers. We might expect therefore that competition from organisms already in possession of the slides would cause heavy mortality in population H, but we would also expect this to occur in the youngest stages from about the second day to the nineteenth day, that is, between the onset of feeding and the appearance of a fully formed black ascidian. This, in fact, does not appear to have happened and during this phase of development the survival curve follows closely that of the populations which settled early in the life of the community. The divergence between the curves does not take place until the point at which the animals had reached the opaque black stage. We must, therefore, look for another explanation, and this may be found in the nature of the community which developed in association with this population. The usual community developing in association with these populations comprised green algae (*Enteromorpha*), cirripedes (*Balanus*), serpulid polychaetes (*Eupomatus*), colonial ascidians (*Didemnum candidum* Savigny), *Diplosoma macdonaldi* Herdman and *Symplegma viride* (Herdman), lamellibranchs (probably *Ostrea equestris* Say) and a large errant fauna of amphipods, copepods, turbellarians, polychaete annelids, herbivorous gastropods (on the algae) and occasionally nudibranchs. The community in association with population I differed markedly from all others in that a large population of pyurid ascidians (*Microcosmus exasperatus* Heller) developed and was already in evidence by the time the *Ascidia nigra* were 20 days old; they eventually became the dominant organism in this community. Pyurid ascidians in general belong to a later stage in the natural succession of inshore sessile communities in Jamaica (unpublished observations), and may in this case have played some part in displacing the *A. nigra,* either by competition for food or by squeezing them off their attachment bases.

Conclusions

The general picture emerging from these data on *Ascidia nigra* is that survival of between 2% and 3% may be normal for a population at 7 feet after the first 6 weeks of life. All the artificial populations and one of the natural populations reared at 7 feet below the sea surface exhibited this pattern of survival, but this is not true of populations reared at the four-foot level. Of four populations reared at this level, two left no survivors and two left between 12% and 18% survivors.

It is probable that the two populations which left no survivors were affected adversely by dense algal growth, but until more information is available, we can only speculate on the high survival of the other two. This extreme divergence between growth of populations reared at the four-foot level, and the smaller divergence between population H and the other populations reared at 7 feet, does, however, focus attention on the effects of environmental differences on survival rates of developing ascidians. These differences can be considered as having dimensions in both space and time. On the one hand, divergence between populations at the four-foot level is assumed to be the result of seasonal differences in the settlement of other organisms at that level. On the other hand, the difference noted between populations at different vertical levels is probably indirectly due to differences in illumination; this results in different biotic communities developing in association with, and in competition with, the ascidians. In both cases it is assumed that differences in the associated community are responsible for the differences in survival, but the community differences are due in the first instance to seasonality and in the second to spatial separation. Similarly, it is suggested that the divergence between the two natural populations, H and I, is a biotic effect due to direct competition between two species of ascidians. These are obviously very complex problems and we hope to progress further with them in the next few years.

At the outset of this work it was thought possible that there might be some correlation between mortality rate and the stage of development of the young ascidian. There is no clear-cut evidence of such a correlation from these data, and the most that can be said is that the highest mortality occurs before the animal becomes completely black, about the nineteenth day. The causes of mortality in the young ascidian are varied. Some zooids are undoubtedly eaten by flatworms and occasionally by young polychaete worms; others have been found dead on the slide with numerous ciliate protozoa inside the empty test. Other ascidians appear to starve to death in the shadow of a barnacle; ascidians growing very close to barnacles have been observed to shrivel slowly from one inspection to another, as if they were unable to obtain sufficient food in competition with the barnacle. A further cause of mortality is due to spatial competition with colonial ascidians such as *Diplosoma macdonaldi, Didemnum candidum* and *Symplegma viride;* all these species have been seen to smother young *A. nigra* as the colony spreads across the slide. Mention has already been made of the possibility that dense mats of algal growth may be responsible for ascidian mortality, presumably by preventing an adequate flow of food from reaching the ascidian.

This work was originally supported by a grant from the Nuffield Foundation, to whom grateful acknowledgment is made. Much of the original observation on the early developmental stages was made at the Bermuda Biological Laboratory; I am grateful to the Director and staff of that laboratory for their help there, and to the National Science Foundation and the University College of the West Indies for grants making the visit possible. I am also grateful to Mr. B. Wade who checked many of the populations during the summer of 1961.

SUMMARY

1. A time scale for the development of *Ascidia nigra* is given, from fertilization of the egg to the completion of black pigment formation. Embryonic and larval development are completed in less than 24 hours, and a functional ascidian is developed within 48 hours of larval settlement. Pigment first appears about the thirteenth day and the animal is completely black by the eighteenth or nineteenth day. The whole developmental process is completed about six times faster than in temperate-water ascidians at temperatures about 11° C. less.

2. The survival of populations of ascidians has been followed from settlement to the end of the sixth week of life. Most populations left only 2% to 3% survivors, but some left none and one as many as 17.5% survivors. These differences are discussed and are assumed to be due to differences in the associated biotic community arising from differences in illumination and season of growth.

3. Death is due to a number of factors, including predation by flatworms and polychaete annelids, competition for food and for space with other sessile animals or algae, and possibly in some cases protozoan infection.

LITERATURE CITED

Berrill, N. J., 1935. Studies in Tunicate development. III. Differential retardation and acceleration. *Phil. Trans. Roy. Soc. London, Ser. B,* **225**: 255–336.

Berrill, N. J., 1947. The development and growth of *Ciona. J. Mar. Biol. Assoc.,* **26**: 616–625.

Goodbody, I., 1961. Continuous breeding in three species of tropical ascidian. *Proc. Zool. Soc. London,* **136**: 403–409.

Goodbody, I., 1962. The biology of *Ascidia nigra* (Savigny). I. Survival and mortality in an adult population. *Biol. Bull.,* **122**: 40–51.

Grave, C., 1925. Preliminary report on development and behavior of larval ascidians and periodicity in spawning of certain marine invertebrates. *Carnegie Inst. Wash. Year Book,* **24**: 224–228.

Grave, C., 1935. Metamorphosis of ascidian larvae. *Publ. Carnegie Inst. Wash.* No. **452**: 209–292.

Grave, C., and P. A. Nicoll, 1940. Studies on larval life and metamorphosis in *Ascidia nigra* and species of *Polyandrocarpa. Papers Tortugas Lab. Carnegie Inst.,* **32**: 1–46.

Millar, R. H., 1951. The development and early stages of the ascidian *Pyura squamulosa* (Alder). *J. Mar. Biol. Assoc.,* **30**: 27–31.

Millar, R. H., 1954. The development of the ascidian *Pyura microcosmus* (Savigny). *J. Mar. Biol. Assoc.,* **33**: 403–407.

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CHLORIDE EXCHANGES IN RAINBOW TROUT (SALMO GAIRDNERI) ADAPTED TO DIFFERENT SALINITIES[1]

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The water and salt balance mechanisms used by teleost fishes have attracted considerable interest for almost a century (recent general reviews are: Black, 1957; Gordon, 1963; Prosser and Brown, 1961; Shaw, 1960). While a great deal of work has been done on fishes maintained under fairly constant conditions in either fresh or sea water, very little effort has been directed at studies of the changes in the basic regulatory mechanisms which occur when fishes encounter environmental changes. Salinity changes are among the commonest of these variations.

The responses of euryhaline teleosts are particularly interesting in this regard, since these forms are usually very good osmotic and ionic regulators. The relative constancy of the concentration of their internal medium means that, when these fishes enter environments of different salinities, they maintain across their integuments osmotic gradients of different magnitudes and even directions. These changes in osmotic gradients in turn mean that, in order for constancy of internal concentration to continue, the fishes must change either or both the fluxes and effective permeabilities for water and at least sodium and chloride across their major exchange pathways—the gills, gut and kidneys.

The three-spined stickleback (*Gasterosteus aculeatus*) seems to be the only euryhaline fish for which even limited data are available on the mechanism of adaptation to changes in trans-integumentary osmotic gradients. Heuts (1942) found that the chloride concentration in cloacal excreta increased about 20 times over fresh-water levels in sticklebacks in ⅓ sea water. Mullins (1950) presented data which he interpreted as indicating that sticklebacks increased their rate of drinking of external medium as the concentration of that medium increased. He also thought that the permeability of the gills to specific ions, especially potassium, changed.

The rainbow trout (*Salmo gairdneri*) is a euryhaline teleost which survives well in both fresh water and the sea. It is a very good osmotic and ionic regulator (Busnel, 1942; Busnel and Drilhon, 1944, 1946; Houston, 1959, 1961; Parry, 1960, 1961). This paper describes a study of the rates of exchange of body chloride by rainbow trout acclimatized to fresh water and sea water, and to several intermediate salinities. The results are interpreted as indicating that the change-over from hyper-osmotic regulation in fresh water to hypo-osmotic regulation in sea water, at least in this species, is based in large part on changes in permeability to water of the gills and other permeable portions of the integument.

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A preliminary discussion of some of these results was given by Gordon (1959a).

MATERIALS AND METHODS

Sexually mature two- to three-year-old rainbow trout, of 100–250 gm. weight, were obtained from a commercial fish hatchery. These fish were maintained in individual aquaria in the laboratory. Each aquarium was equipped with aeration and a system whereby circulating water of any salinity between fresh water (dechlorinated Los Angeles tap water) and 100% sea water (equals 32‰ salinity, 960 mOsmoles/liter osmotic concentration) could be supplied continuously for any desired period. Water temperature was maintained at 19 ± 2° C. Photoperiod was that normal for the season. No experiments were done during the autumn breeding season, when salinity tolerance in this species is sometimes markedly decreased. Fish were not fed.

Groups of fish were maintained in fresh water for several days after receipt. Ten days were allowed for acclimatization to each higher salinity used. Transfers were made directly from fresh water to $\frac{1}{7}$ and $\frac{1}{3}$ sea water. Acclimatization to still higher salinities was done stepwise (*e.g.* 10 days in $\frac{1}{3}$ sea water, then 10 days in $\frac{2}{3}$ sea water, then 10 days in $\frac{3}{3}$ sea water).

Estimates of rates of chloride exchange were carried out on groups of five trout acclimatized to fresh water, $\frac{1}{7}$, $\frac{1}{3}$, $\frac{2}{3}$, and $\frac{3}{3}$ sea water. Excepting only $\frac{1}{7}$ sea water, observations were made at each concentration, both on normal, intact fish and on fish which had had their cloacas and urinary papillae tightly ligated. The effectiveness of cloacal ligation was tested at the end of each experiment by pressing on the abdomen of the fish in order to extrude bladder urine or gut contents. In all cases pressures required were so far above those which the fish themselves could have produced that I am confident no leakage occurred during the experiments. All operations on trout were performed with the fish held under water in a piece of smooth cotton cloth. Water used was always that to which the fish were acclimatized.

Radioactive chlorine-36 was used as a tracer for chloride movements. This isotope was obtained as carrier-free HCl^{36} with specific activity of about 500 μc./gm. chloride, from Oak Ridge. This solution was neutralized with NaOH and diluted to produce a final injection solution of about 160 mM $NaCl^{36}$. Final activities of injection solutions were in the range 20–30 μc./ml.

$NaCl^{36}$ solutions were injected intraperitoneally. Total injection volumes were in the range 0.08–0.15 ml., calculated to produce doses of approximately 15 μc./kg. The fish were then transferred to individual small closed plastic aquaria, each containing 1500 ml. of water of the appropriate concentration and supplied with aeration. Two-ml. aliquots of the external medium were taken at one-hour intervals for 6 hours. The fish were then sacrificed, a blood sample was taken by heart puncture, and they were re-weighed to the nearest gram.

The aliquots of external medium, also duplicate 25-μl. aliquots of blood samples, were evaporated to dryness on aluminum planchets. Times required for the occurrence of 5120 counts per sample were determined with a Nuclear-Chicago Model D-47 thin end window (0.1 mg./cm.^{2}) gas flow counter connected with an automatic sample changer, scaler and printing timer. Background was stable at 15 cpm., resulting in assay precision of ±2–3%. Absolute activity of samples was determined by comparison with standards of known activity made from the original HCl^{36} solutions obtained from Oak Ridge. These standards were dissolved in appropriate volumes of the sea water dilutions used, and prepared and counted in the same way as unknowns.

Total chloride in blood samples was determined on duplicate 0.1-ml. aliquots with an Aminco-Cotlove automatic chloride titrator. Precision was 2–3 meq./l. of whole, hemolyzed blood. Blood specific activity for each fish at the end of each experiment was calculated from these data and the radioactivity assays.

Measured rates of appearance of Cl^{36} were converted to μc. Cl^{36}/kg. fish/hr. for fish with a uniform specific activity of their blood one hour after injection of 0.50 μc. Cl^{36}/meq. total Cl in blood. These adjustments were required since there were variations in radioactivity of body chloride in each fish in each experimental group, which resulted from errors in initial weighing and variations in amount of total body chloride.

Procedure for adjusting rate measurements was as follows: No recycling of radioactivity into the fish was assumed, as was a steady-state for the chloride content of the fish. The fraction of the injected dose which appeared in the medium during the 6 hours duration of the experiment was calculated. Those few fish were discarded which had exchanged more than about 20% of the injected dose (these probably had been damaged in handling), as were those with such low blood chloride specific activities that it was apparent the initial injection had not been successful. Rates of exchange of radioactivity were assumed to have been proportional to blood chloride specific activity. Blood chloride specific activity one hour after the initial injection was assumed to have been equal to: measured final specific activity/fraction of injected isotope dose remaining in fish at end of experiment. A factor was then calculated for each fish which would adjust the calculated blood specific activity one hour after the injection to 0.50 μc./meq. Cl. The measured rates of appearance of radioactivity for each fish, in μc./kg./hr., were then multiplied by this factor.

Data for the first hour following the injections were not used. Mean rates of appearance, also standard errors, for each of the remaining five hours of each experiment were calculated. Excepting only the experiments in fresh water, there were no statistically significant differences within the sets of five hourly means for each experiment.

Cumulative rates of isotope appearance for the five usable hours of each experiment were next calculated for each fish in each group. Means and standard errors for these cumulative rates were calculated and comparisons between the various groups made by analysis of variance.

As a check on the osmotic and ionic regulatory abilities of the rainbow trout used in this work, blood samples were taken by heart puncture from additional groups of animals, acclimatized to various salinities under the same conditions as the experimental animals. No samples were taken during the breeding season. Freezing point depressions were determined by the method of Ramsay and Brown (1955), with a precision of ± 0.02° C. Chloride concentrations were determined on the automatic chloride titrator as described above.

RESULTS

Plasma osmotic and chloride concentrations in rainbow trout acclimatized to various salinities from fresh water to ⅔ sea water are shown in Figure 1. No seasonal changes were noted. These results agree with those of the other workers cited in the introduction. Plasma chloride concentration closely parallels osmotic concentration. Regulation of these concentrations is not perfect, but it is quite good. Fish in fresh water maintain osmotic gradients across their integuments almost twice as large as those maintained by fish in 1/7 sea water. These gradients act to move water into the animal. Fish in ⅔ sea water maintain osmotic gradients, in the opposite direction, 30 to 50 times larger than those maintained by fish in ⅓ sea water.

Table I and Figure 2 present the results of the adjusted measurements of cumulative rates of Cl^{36} appearance from variously acclimatized trout, both unoperated fish and those with ligated cloacas and urinary papillae ("ligated fish"), during the period February through May. Table II summarizes the results of the statistical analyses of these data. The measurements on ligated fish are interpreted as estimates of the rates of chloride exchange across only the gills and other permeable parts of the integument. The differences between these rates and those for unoperated animals are considered estimates of the rates of chloride loss by way of the gut and, especially, the kidneys.

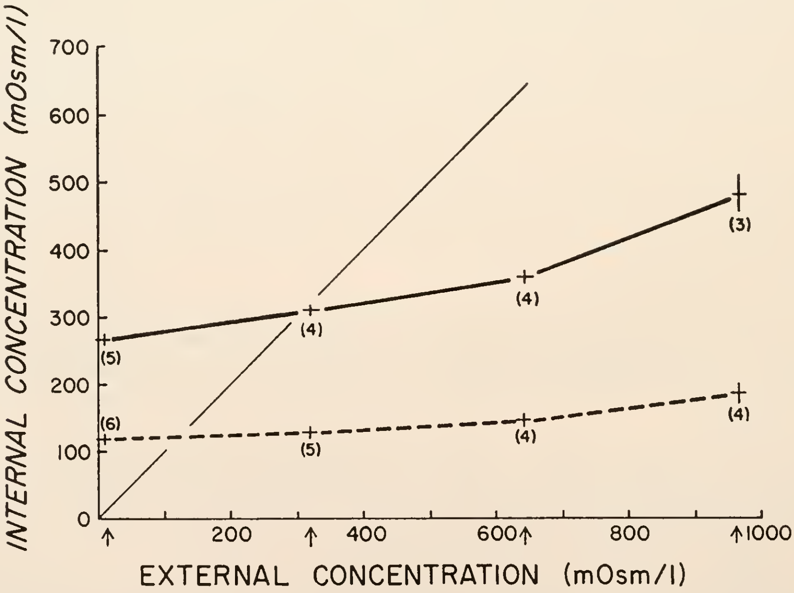

FIGURE 1. Osmotic and chloride concentrations of plasma vs. osmotic concentration of external medium, in rainbow trout acclimatized to various salinities. Horizontal lines indicate means of observations on indicated numbers of fish; vertical lines ± 2 S.E.'s. Solid line: plasma osmotic concentration (freezing point depression); dashed line: plasma chloride concentration. Diagonal line is line of equality. Fish acclimatized to each medium for at least 10 days. All experiments at 19 ± 2° C. Arrows along abscissa indicate osmotic concentrations of fresh water, ⅓, ⅔ and ⅗ sea water.

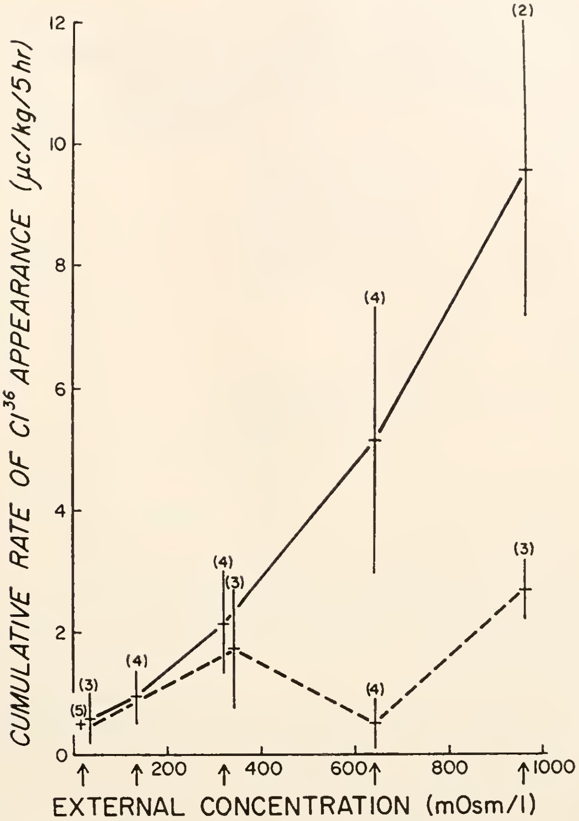

FIGURE 2. Cumulative rate of appearance of Cl^{36} from rainbow trout acclimatized to various salinities at 19 ± 2° C. between February and May. See text for details. Solid line: unoperated fish; dashed line: fish with sewn cloacas and urinary papillae. Symbols as Figure 1.

Rates of total chloride exchange for unoperated animals in hypo-osmotic media (fresh water and $\frac{1}{7}$ sea water) are all statistically significantly lower than rates for unoperated animals in all of the hyper-osmotic media. Total exchange rates increased as external osmotic and chloride concentrations rose. Rates for fish in $\frac{2}{3}$ sea water are not statistically significantly different from rates for fish in $\frac{1}{3}$ sea water. The difference in rates between $\frac{2}{3}$ and $\frac{3}{3}$ sea water is significant.

Fish in fresh water are indicated to have exchanged chloride about one-half as rapidly as fish in $\frac{1}{7}$ sea water. The external chloride pool in the fresh-water experiments was sufficiently small so that this result may have been affected by recycling of isotope by the fish. There was a statistically significant continuous secular decline in rate of change of external isotope concentration in the fresh-water experiments. The rate of isotope appearance during the second hour of the fresh-water series was the same as the rate determined for the same period in $\frac{1}{7}$ sea water. The rate constants calculated for these two groups by analyses of the data as two compartment systems (Solomon, 1960, p. 130) were also the same. The true

TABLE I

Cumulative rates of Cl^{36} appearance from rainbow trout (blood specific activity 0.50 μc. Cl^{36}/meq. Cl at one hour; 19 ± 2° C.)

State of acclimatization	Rates of Cl^{36} appearance (μc./kg./5 hr.) [$\bar{X}$ ± S.E. (N)]	
Unoperated fish	*February–May*	*July–August*
FW	0.51 ± 0.06 (5)	—
1/7 SW	0.98 ± 0.24 (4)	—
1/3 SW	2.16 ± 0.42 (4)	1.75 ± 0.16 (4)
2/3 SW	5.16 ± 1.09 (4)	3.68 ± 0.59 (4)
3/3 SW	9.6 ± 1.2 (2)	2.24 ± 0.17 (3)
Fish with ligated cloaca and urinary papilla		
FW	0.61 ± 0.22 (3)	—
1/3 SW	1.75 ± 0.48 (3)	—
2/3 SW	0.54 ± 0.20 (4)	—
3/3 SW	2.72 ± 0.24 (3)	—

cumulative rate for fresh water is therefore probably similar to the rate for 1/7 sea water.

Fish in 3/3 sea water exchanged chloride approximately 4½ times more rapidly than fish in 1/3 sea water, about twice as rapidly as fish in 2/3 sea water.

The pattern for ligated fish is statistically significantly different from that for unoperated fish only in 2/3 and 3/3 sea water. The results for the ligated group in fresh water duplicated those for unoperated fish both in time sequence and cumulatively. This indicates that virtually all chloride exchange in trout in fresh water occurs across the gills and other permeable parts of the integument. The same appears to be true for fish in 1/3 sea water.

The situation in strongly hyperosmotic media appears to be different. Rate of chloride exchange by ligated fish in 2/3 sea water was only about 10% of that for unoperated fish. In 3/3 sea water this fraction was near 25%. The rate for ligated fish in 2/3 sea water is not statistically significantly lower than the rate for ligated fish in 1/3 sea water. The rates for ligated fish in 2/3 sea water and 3/3 sea water differ significantly. The rates for ligated fish in 1/3 sea water and 3/3 sea water are

TABLE II

*"F" values resulting from analysis of variance comparisons between groups of rainbow trout, February through May**

Unoperated fish	FW	1/7 SW	1/3 SW	2/3 SW	3/3 SW	Ligated fish
3/3 SW	396***	297***	133***	13.0***	—	3/3 SW
2/3 SW	12.1***	9.59**	4.54	—	61.7***	2/3 SW
1/3 SW	12.4***	5.35**	—	2.50	1.40	1/3 SW
1/7 SW	3.95	—	—	—	—	1/7 SW
FW	—	—	2.20	0.30	55.6***	FW

* "F" values with no asterisks indicate that the groups compared are not statistically significantly different. Two asterisks indicate significance at the 5% level, three asterisks at the 1% level.

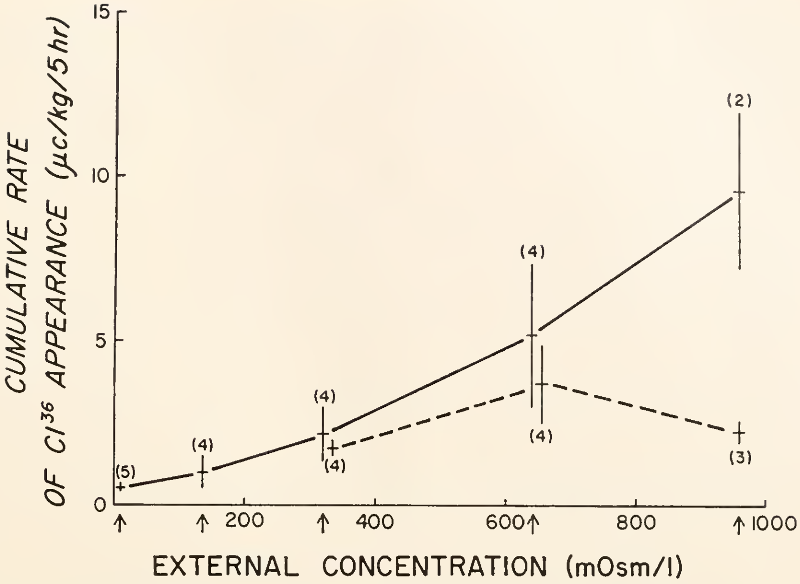

FIGURE 3. As Figure 2, but unoperated fish at different times of year. Solid line: experiments between February and May; dashed line: experiments in July and August.

not statistically significantly different. These results indicate that trout in hyperosmotic media may exchange by way of the permeable parts of their integument only a small fraction of the total amount of chloride they take in by drinking of external medium. The amount of chloride exchanged across these permeable areas (presumably primarily the gills) does not change greatly, even though the osmotic gradients maintained by the fish increase tremendously.

Table I and Figure 3 present the results of the adjusted measurements of cumulative rates of Cl^{36} appearance from variously acclimatized unoperated trout during the periods February through May and July through August. A comparison of these two periods was made because Gordon (1959b) had found the osmoregulatory abilities of the brown trout (*Salmo trutta*) to be significantly lessened during July and August, as compared with the rest of the year.

The July-August results differ from the February-May results only for fish in 3/3 sea water. Trout acclimatized to 3/3 sea water in summer exchanged chloride only 1/4 as rapidly as similarly treated fish in spring. The lack of similar differences for trout in 1/3 and 2/3 sea water makes it improbable that this single difference actually reflects seasonal changes in the fish. It seems much more probable that the difference was due to differences in experimental manipulation of the two groups, the summer group having been less shocked and damaged by handling than the spring group.

DISCUSSION

Two major difficulties complicate the interpretation of the results of experiments such as these. First, fishes are notoriously sensitive to handling. This is due not only to the changes in internal hormonal concentrations resulting from the stress imposed by experimental manipulations, but also to mechanical changes in the permeability of their integument due to rubbing off of mucus, scratches, etc. The usual result of these effects is the induction, apparently only in fish in hyper-osmotic media, of a more or less severe state of "laboratory diuresis." This condition is characterized by the production of abnormally large volumes of urine of abnormally high salt content. The condition appears almost immediately after handling of fish (Forster and Berglund, 1956; Holmes, 1961).

It is probable that the increased rates of chloride exchange shown by unoperated rainbow trout in ⅔ and 3/3 sea water were due in large part to the occurrence of progressively more severe cases of laboratory diuresis. The considerable variability of most of the measurements for such groups fits in with this interpretation. The low result for the group in 3/3 sea water in summer is probably the most reliable of the four presented. It seems most reasonable, therefore, to consider all of these results as upper limits for the total rates of chloride exchange by the fish involved.

The second complication is that an unknown and perhaps variable fraction of the measured rates of isotope appearance may have been due to the physical exchange of unlabelled for labelled Cl atoms ("exchange diffusion") and not have represented actual unidirectional ionic fluxes produced by particular transport processes. None of the data required for estimation of the rates at which exchange diffusion may have taken place are available (Cooperstein and Hogben, 1959). It is therefore impossible to say with certainty that the rates measured for ligated fish represent either (a) rates of passive outward diffusion of chloride across the gills, etc., of trout in hypo-osmotic media, or (b) rates of active excretion of chloride across the same tissues of fish in hyper-osmotic media. The rates determined are, however, upper limits for the rates at which these processes could occur.

Even with these qualifications, several inferences still seem reasonable. Note again that in both hypo-osmotic and hyper-osmotic media neither total chloride exchanges nor integumentary chloride exchanges varied proportionally with the magnitudes of the trans-integumentary osmotic gradients maintained by the fish.

Assume that the surface/volume ratio for the trout is constant in all salinities. Let the null hypothesis be that the diffusion coefficients for water across the permeable parts of the integument of rainbow trout are constant in all media. Increases in magnitude of osmotic gradients should, in this situation, produce proportional increases in rates of water movement. Assuming the experimental fish were in steady states with regard to water and salt, the implications would be: (a) the more dilute the external hypo-osmotic medium, the greater the rate of urine production and, with fairly constant urinary salt content such as usually occurs in fishes, the more rapid the rate of urinary salt loss; (b) the more concentrated the external hyper-osmotic medium, the greater the rate of drinking of that medium and the more rapid the rate of active salt excretion by the gills (osmotic gradients maintained by trout in 3/3 sea water were a minimum of 30 times larger than gradients maintained by fish in ⅓ sea water; there is three times as much salt per unit volume in 3/3 sea water as there is in ⅓ sea water; trout in 3/3 sea water might, therefore, be expected to excrete chloride 90 or more times faster than fish in ⅓ sea water).

The data agree with neither of these predictions. It seems most improbable that the amount of integumentary exchange diffusion in hyper-osmotic media would change sufficiently to mask changes in rates of active excretion of the magnitudes required by the model. It is probable, therefore, that a most important part of the process of salinity acclimatization in rainbow trout is a reduction in integumentary permeability to water. These permeability reductions are more or less proportional to the magnitudes of transintegumentary osmotic gradients, whatever the direction of these gradients.

This conclusion implies that adaptation to different salinities does not necessarily impose on rainbow trout markedly different energy requirements for osmoregulatory purposes. It is possible that much of the adjustment is taken care of by changes in the physical state of the permeable areas of the integument, changes mediated, perhaps, by a neurohypophysial hormone (Hays and Leaf, 1962).

Support for these results and inferences can be derived from some calculations. Assume that the cumulative rate of chloride exchange by trout in fresh water was actually that measured for fish in $\frac{1}{7}$ sea water. Assume also that the specific activity of the chloride exchanged was the same as the adjusted specific activity used for the blood, *i.e.,* 0.50 μc. Cl^{36}/meq. total Cl. The total rate of chloride loss from trout in fresh water, on this basis, was about 9.6 meq. Cl/kg./24 hr. Krogh (1937) estimated that small rainbow trout in fresh water absorbed Cl from their environment at the rate of 7.2 meq. Cl/kg./24 hr. Phillips *et al.* (1958), working with small brook trout (*Salvelinus fontinalis*), measured a total rate of Cl uptake from fresh water of 3 m*M* Cl concentration of about 1.8 meq. Cl/kg./24 hr.

The data of Krogh (1937) and Holmes (1961) indicate that urinary Cl losses in fresh water should account for almost half of the total losses. The present data do not indicate this.

Other euryhaline species, such as the common eels (*Anguilla* spp.) drink 30–200 ml./kg./24 hr. when acclimatized to sea water (Smith, 1930; Keys, 1933). Assuming no exchange diffusion and a specific activity of body Cl of 0.50 μc./meq., the maximum measured rate of total Cl exchange in $\frac{3}{3}$ sea water (February–May) is equivalent to the ingestion by the trout of about 200 ml./kg./24 hr. The rate for July–August fish is equivalent to about 40 ml./kg./24 hr.

Similar calculations for trout in $\frac{1}{3}$ and $\frac{2}{3}$ sea water give drinking rates of approximately 40 ml./kg./24 hr. and 100 ml./kg./24 hr., respectively, between February and May. Summer fish are similar.

SUMMARY

1. Estimates of rates of exchange of body chloride, both total exchanges and exchanges across the integument and by way of the gut and kidneys, have been made in rainbow trout (*Salmo gairdneri*) acclimatized to various salinities between fresh water and sea water (salinity 32‰). Radioactive chlorine-36 was used as a tracer of chloride movements.

2. Neither total Cl exchanges nor integumentary exchanges varied in proportion with changes in the magnitude of the transintegumentary osmotic gradients maintained by the fish. This result is interpreted as indicating that changes in the permeability to water of the integument (probably primarily the gills) are an important part of the salinity adaptation process in rainbow trout.

3. Laboratory diuresis and exchange diffusion of chloride are discussed as possible complications in this interpretation.

LITERATURE CITED

Black, V. S., 1957. Excretion and osmoregulation. *In:* The Physiology of Fishes (M. E. Brown, ed.), **1**: 163–205.

Busnel, R. G., 1942. Recherches de physiologie appliquées à la pisciculture: à propos de la migration de la truite arc-en-ciel. *Bull. Français Pêche Piscicult.*, **15**: 45–65.

Busnel, R. G., and A. Drilhon, 1944. Modifications de la concentration moléculaire du milieu intérieur de *Salmo iridaeus* au cours de la croissance, en rapport avec l'adaptation aux changements de salinité. *C. R. Soc. Biol.*, **138**: 334–336.

Busnel, R. G., and A. Drilhon, 1946. Recherches sur la physiologie des Salmonides. Bull. Inst. Océanogr. (Monaco), No. **893**: 1–7

Cooperstein, I. L., and C. A. M. Hogben, 1959. Ionic transfer across the isolated frog large intestine. *J. Gen. Physiol.*, **42**: 461–473.

Forster, R. P., and F. Berglund, 1956. Osmotic diuresis and its effect on total electrolyte distribution in plasma and urine of the aglomerular teleost, *Lophius americanus*. *J. Gen. Physiol.*, **39**: 349–360.

Gordon, M. S., 1959a. Rates of exchange of chloride ions in rainbow trout (*Salmo gairdneri*) acclimated to various salinities. *Anat. Rec.*, **134**: 571–572.

Gordon, M. S., 1959b. Ionic regulation in the brown trout (*Salmo trutta* L.). *J. Exp. Biol.*, **36**: 227–252.

Gordon, M. S., 1963. Water and salt balance in fishes and amphibians. *Handbook of Physiology*, **4** (in press).

Hays, R. M., and A. Leaf, 1962. The state of water in the isolated toad bladder in the presence and absence of vasopressin. *J. Gen. Physiol.*, **45**: 933–948.

Heuts, M. J., 1942. Chloride-excretie bij *Gasterosteus aculeatus* L. *Ann. Soc. Roy. Zool. Belg.*, **73**: 69–72.

Holmes, R., 1961. Kidney function in migrating salmonids. *Ann. Rept. Challenger Soc.*, **3**, No. 13: 23.

Houston, A. H., 1959. Osmoregulatory adaptation of steelhead trout (*Salmo gairdneri* Richardson) to sea water. *Canad. J. Zool.*, **37**: 729–748.

Houston, A. H., 1961. Influence of size upon the adaptation of steelhead trout (*Salmo gairdneri*) and chum salmon (*Oncorhynchus keta*) to sea water. *J. Fish. Res. Bd. Canada*, **18**: 401–415.

Keys, A., 1933. The mechanism of adaptation to varying salinity in the common eel and the general problem of osmotic regulation. *Proc. Roy. Soc. London, Ser. B*, **112**: 184–199.

Krogh, A., 1937. Osmotic regulation in fresh water fishes by active absorption of chloride ions. *Zeitschr. vergl. Physiol.*, **24**: 656–666.

Mullins, L. J., 1950. Osmotic regulation in fish as studied with radioisotopes. *Acta Physiol. Scand.*, **21**: 303–314.

Parry, G., 1960. The development of salinity tolerance in the salmon, *Salmo salar* (L.) and some related species. *J. Exp. Biol.*, **37**: 425–434.

Parry, G., 1961. Osmotic and ionic changes in blood and muscle of migrating salmonids. *J. Exp. Biol.*, **38**: 411–427.

Phillips, A. M., Jr., H. A. Podoliak, R. F. Dumas and R. W. Thoesen, 1958. Absorption of dissolved chloride by brook trout. Cortland (N.Y.) Fish Hatchery Fish Res. Bull., No. 22: 8–16.

Prosser, C. L., and F. A. Brown, Jr., 1961. Comparative Animal Physiology. W. B. Saunders Co., Philadelphia; second edition.

Ramsay, J. A., and R. H. J. Brown, 1955. Simplified apparatus and procedure for freezing-point determinations upon small volumes of fluid. *J. Sci. Instr.*, **32**: 372–375.

Shaw, J., 1960. The mechanisms of osmoregulation. *In:* Comparative Biochemistry (M. Florkin and H. S. Mason, eds.), **2**: 471–518.

Smith, H. W., 1930. The absorption and excretion of water and salts by marine teleosts. *Amer. J. Physiol.*, **93**: 480–505.

Solomon, A. K., 1960. Compartmental methods of kinetic analysis. *In:* Mineral Metabolism (C. L. Comar and F. Bronner, eds.), **1A**: 119–167.

THE EFFECT OF LITHIUM AND O-IODOSOBENZOIC ACID ON THE EARLY DEVELOPMENT OF THE SEA URCHIN EGG

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In 1892 C. Herbst observed that the addition of lithium ions to sea water changes the determination of the sea urchin egg in a vegetal direction, *i.e.,* the development of the endoderm becomes accentuated, whereas the differentiation of the ectoderm is suppressed, leading to a vegetalization of the larva. This specific effect of lithium has been utilized in a number of important investigations on developmental physiology; for references, see, for example, Gustafson (1954), Hörstadius (1935, 1939, 1949), Lindahl (1936, 1941), Runnström (1954), von Ubisch (1953).

The vegetalizing action of lithium becomes apparent in an exogastrulation of the larva, *i.e.,* there is no invagination of the gut; the ciliation of the larva is also reduced. These aberrant morphological characteristics are not manifested, however, until a rather late stage of development, whereas the treatment, which gives rise to the changes referred to above, may be applied in immediate connection with fertilization. The direct effect of lithium on the larva during the actual period of treatment does not seem, as yet, to have been studied in detail.

Animalization (the opposite of vegetalization), which is manifested as an increase in the development of the ectoderm and a reduced differentiation of the endoderm, has likewise been achieved by various treatments (*cf.* Lindahl, 1941; Runnström, 1954). These so-called animalized larvae show a strong ciliation and a suppressed development of the gut. It has recently been demonstrated that o-iodosobenzoic acid (IBA) exerts a strong animalizing action on sea urchin larvae (Bäckström, 1953; Runnström and Kriszat, 1952a, 1952b); the treatment with IBA is also applied during the early development of the larva, during the first hours after fertilization.

In 1952, the author observed that lithium interferes with cell division, and also the movements of the chromosomes were found to be affected when lithium ions in concentrations of 0.05–0.06 M were present in the sea water. Owing to other investigations in progress, these first observations on the cytological effect of lithium were not followed up until 1959, when the present work was started.

MATERIAL AND METHODS

The present study was carried out at Stazione Zoologica in Naples, where *Paracentrotus lividus* and *Psammechinus microtuberculatus* served as material. Experiments have also been made at Biologisk Stasjon, Espegrend, Norway, with eggs and sperm from *Echinus esculentus* and *Strongylocentrotus droebachiensis,* and at Kristinebergs Zoologiska Station, Fiskebäckskil, Sweden, where the gametes from *Echinocardium cordatum* and *Psammechinus miliaris* were used.

In the experiments with lithium the concentrations varied from 0.03 *M* to 0.09 *M*. The concentrations of o-iodosobenzoic acid used varied between 5×10^{-5} *M* and 10^{-3} *M*.

The experimental treatment with lithium and IBA was applied according to several different methods. In most experiments the active substances were added to batches of the egg suspension within 5–10 minutes after insemination. At different time intervals after the first treatment was started, new batches of eggs from the fertilized control were transferred to the lithium and IBA. In other series, lithium and IBA were added to the larvae when they were passing through a certain cell phase, *e.g.* prophase or metaphase, or when they were just completing cell division, *e.g.* from the 4- to the 8-cell stage.

In most of the experiments the treatment with the active substances was interrupted at the 64–128-cell stage, and the larvae were transferred to pure sea water; in other series, the larvae were reared in the presence of lithium or IBA through the hatching stage.

The cultures with cleaving larvae were counted at different time intervals after insemination; the percentages of cleavage given in this paper are based on counts of 200–250 larvae. The counts were made either in the living state or after fixation in Carnoy's fluid or in 4% formol. When the counts were made on the living cultures, the control and the cultures treated were in most experiments inseminated successively at intervals of 5 minutes, which enables the counts to be made at the same point of time after insemination.

Studies of the chromosomes and the nuclear phases were carried out after fixation of the larvae in Carnoy's fluid and staining with aceto-carmine or aceto-orcein.

RESULTS

The effect of lithium on the rate of cleavage

When fertilized eggs were transferred to sea water containing lithium, a considerable retardation in the rate of cleavage was observed. In experiments where the inseminated eggs were subjected to lithium already before the sperm and egg nuclei had fused, this retardation became particularly evident, often resulting in a complete blocking of the first cell division. This was especially noticeable when the concentration of lithium was kept rather high, *i.e.*, 0.06–0.09 *M*.

A certain variation in sensitivity to lithium between the different species tested was observed and, for example, in *Strongylocentrotus droebachiensis* and *Echinus esculentus*, species which develop at a rather low temperature, and consequently the first cleavage requires three hours or more to be completed, the blocking effect on the fusion of the pronuclei was more pronounced than in *Paracentrotus*. The latter species develops more rapidly and at a much higher temperature. The retardation noted at the first cleavage is evident also during the ensuing development.

In other experiments the treatment with lithium was started at different time intervals after the first cell division, and also in these experiments the retarding effect of lithium on cleavage was striking. The sensitivity to lithium was tested in consecutive experiments up to the 64- to 128-cell stage, when it becomes difficult to observe the single cell divisions.

The effect of o-iodosobenzoic acid on the rate of cleavage

Experiments similar to those described with lithium were carried out also with o-iodosobenzoic acid. Most of these experiments were made at the same time as those with lithium; the same egg material and the same control were used for both series.

In contrast to lithium, IBA was found to effect a general increase in the rate of cleavage. As in the experiments with lithium, treatment with IBA was started before the first cell division, and at different intervals during cleavage, *i.e.,* at the 2-, 4-, 8-, 16-, 32-, and 64-cell stages. The length of treatment was also varied as in the lithium experiments. Thus, the treatment was stopped, for instance, after the 16-cell stage or at the 64- to 128-cell stage. Experimental series were also carried out in which the treatment was not interrupted until after the larvae in the control had begun to hatch.

More than 200 experiments, in which the larvae were reared over hatching, were made in the course of the present investigation. In many of these experiments the development was followed up to the pluteus stage. Though the experiments were carried out with six species of sea urchins, under varied and different experimental conditions, the results were practically uniform. The results of a representative experiment with eggs and sperm from *Paracentrotus lividus* are referred to in Table I.

TABLE I

Eggs and sperm from Paracentrotus lividus. Temperature 18.5° C. (1) Control. The eggs were inseminated with 10^6 spermatozoa per ml. Twenty minutes after insemination eggs from the control were transferred to: (2) 0.06 M LiCl, and (3) 3.5×10^{-4} M IBA

45 minutes after insemination:

No cleavage was observed in either (1), (2) or (3)

58 minutes after insemination:

(1) 90% uncleaved, 10% 2-cell
(2) 100% uncleaved
(3) 40% uncleaved, 60% 2-cell

76 minutes after insemination:

(1) 3% uncleaved, 97% 2-cell
(2) 7% uncleaved, 93% 2-cell
(3) 97% 2-cell, 3% 4-cell

90 minutes after insemination:

(1) 99% 2-cell, 1% 4-cell
(2) 3% uncleaved, 97% 2-cell
(3) 85% 2-cell, 15% 4-cell

135 minutes after insemination:

(1) 2% 2-cell, 50% 4-cell, 48% 8-cell
(2) 3% 2-cell, 85% 4-cell, 12% 8-cell
(3) 8% 4-cell, 92% 8-cell

190 minutes after insemination:

(1) 4% 8-cell, 96% 16-cell
(2) 53% 8-cell, 47% 16-cell
(3) 89% 16-cell, 11% 32-cell

The absolute difference in time between the control and the batches of eggs developing in the presence of lithium or IBA is, among other factors, dependent upon the species used, the concentration of the active substance and the temperature. The retardation obtained in the presence of 0.06 *M* lithium was measured to about 70 minutes for the second cleavage of eggs from *Strongylocentrotus droebachiensis* reared at 8° C. The corresponding retardation observed for eggs from *Paracentrotus* at 19.5° C. was recorded to about 12–15 minutes. At 18° C. this difference may amount to 20 minutes or more. The advance in cleavage obtained by treating the larvae in 7×10^{-4} *M* IBA was found to be 20–25 minutes for the second to fourth cleavages of *Paracentrotus* eggs reared at a temperature of 18.4° C.

As has already been pointed out, many factors influence the rate of cleavage. Experiments of the type shown in Table I have been preferred for evaluating the qualitative effect of the substances tested, whereas in experiments where the absolute retarding or enhancing effect was to be studied, the cultures were fixed at the same stage of development and the difference in time was measured.

The influence of lithium and IBA on the fertilization rate

A sensitive method of testing the positive or negative effect of substances present in fertilization experiments is the so-called fertilization rate method (Hagström and Hagström, 1954, 1959).

Because both lithium and IBA were found to exert a strong influence on early development, it was thought to be of interest to investigate the action of these substances also on fertilization.

Lithium and IBA were added to the egg suspensions immediately before insemination, and consequently, the substances acted on both eggs and spermatozoa at the moment of fertilization. After 5, 10, 15, 20, etc., seconds, sodium lauryl sulphate was added to a final concentration of 0.001%, which instantaneously kills the spermatozoa without affecting the eggs (Hagström and Hagström, 1954, 1959). This method makes it possible to count the number of eggs fertilized 5, 10, 15, etc., seconds after insemination.

The concentrations of lithium in the present experiments were 0.04 *M* and 0.06 *M*, and the concentrations of IBA varied between 3×10^{-4} *M* and 10^{-3} *M*.

Lithium in the concentrations tested produced no effect on the fertilization rate. In the experiments with IBA there was also no clear difference recorded when compared with the control series. In some cases, however, there was a slight improvement with IBA in concentrations of about 10^{-3} *M*. No deleterious effects on the processes at fertilization due to either lithium or IBA were observed. This is of importance for the evaluation of the cleavage rate experiments.

The influence of lithium and IBA on hatching

The first developmental period ends when, at hatching, the sea urchin larva breaks the fertilization membrane and can swim freely. The series of events leading up to hatching makes it possible to record a number of objective observations on the rate of development.

As previously mentioned, the duration of treatment varied, and in some experiments it was interrupted at a relatively early stage, *i.e.,* in the 64- to 128-cell stage, whereas in other experiments the active substances were present from a few minutes after insemination until the larvae started to hatch in the control. Many variations were applied within this general scheme of treatment.

In experiments where the larvae were reared in lithium or IBA from inseminanation until the larvae of the control began to hatch, these substances were found definitely to inhibit hatching. It is known that IBA prevents hatching, and that the larvae may then develop inside the fertilization membrane (Runnström and Kriszat, 1952b). The present experiments showed that lithium also tended to arrest hatching. There is, however, a considerable difference between the action of lithium and that of IBA. Lithium not only delays or completely inhibits hatching, but also prevents the formation of cilia and the movements of the larva inside the intact membrane. In IBA the actual rupture of the membrane is obviously impeded, whereas the ciliation of the larva is normally or even more than normally developed. Consequently, in the presence of IBA the larvae acquire a high degree of rotatory activity inside the membranes. This is also in agreement with the fact that the larvae develop more rapidly in IBA; accordingly, the viability of the larvae seems to be high. A typical experiment is referred to in Table II.

In certain experiments with high concentrations of lithium and IBA, hatching was completely arrested, especially when the slowly developing Norwegian species were used.

Experiments in which the active substances were removed at a relatively early stage of development were also carried out. Qualitatively, the same results were gained as in the experiments mentioned above; lithium gives rise to a general decrease in the rate of development, whereas IBA promotes development, with the exception that IBA affects hatching.

The negative effect exerted by IBA on hatching must be ascribed to the fact that the rupture of the fertilization membrane is arrested, and that this process is not correlated with any retardation of the development in general.

Morphological observations on the cleavage stages

The effect of lithium and IBA on the nuclei and chromosomes appeared to be of special importance. Observations were made with the phase contrast microscope on vital material, and, moreover, about 1500 fixations have been prepared during the course of this investigation. The results of the cytological studies will be reported elsewhere. The present paper will deal only with observations on the cleavage pattern of the young larva.

With IBA, cleavage seems to be normal though accelerated. The cleavage furrows are deep, but the blastomeres remain well attached to each other.

The effects observed after treatment with lithium were considerably more complex. Lithium was found to induce a very clear separation of the blastomeres. This deterioration in the contacts between the cells of the cleaving blastulae is likely to affect the transport mechanism within the larva and the exchange of metabolites between the different regions of the embryo, thus interfering with a mechanism which is undoubtedly of the utmost importance to the ensuing development. When the micromeres form, they seem, however, to retain intercellular contact with each other and with the macromeres. This may be due to the fact that the micromeres have a small volume but a relatively large surface area as compared with the macro-

TABLE II

Paracentrotus lividus, eggs from one female. Temperature 18° C. (1) Control. The eggs were inseminated with 10^6 spermatozoa per ml. Twelve minutes after insemination eggs from the control were transferred to: (2) 0.06 M LiCl, and (3) 7×10^{-4} M IBA

9 hours 50 minutes after insemination:

(1) A few ciliated larvae rotating inside the membranes. No hatched larvae.
(2) No ciliation or hatching.
(3) Most larvae ciliated and moving inside the membranes. No hatched larvae.

10 hours 15 minutes after insemination:

(1) Increased ciliation and movements. No hatching.
(2) No ciliation.
(3) Vigorous movements, stronger than in (1). No hatching.

10 hours 40 minutes after insemination:

(1) A few hatched larvae.
(2) No ciliation, no movements or hatching.
(3) Strong rotation inside the membranes. No hatching.

11 hours 10 minutes after insemination:

(1) About 50% hatched larvae.
(2) A few larvae with weak ciliation.
(3) Vigorous movements, a few hatched larvae.

11 hours 55 minutes after insemination:

(1) About 80% hatched larvae.
(2) About 25% with weak rotating movements. No hatching.
(3) Vigorous movements, about 40% hatched larvae.

12 hours 20 minutes after insemination:

(1) 95% hatched larvae.
(2) No hatching.
(3) About 60% hatched larvae.

12 hours 55 minutes after insemination:

(1) 100% hatched larvae.
(2) No hatching, weak rotation inside the membranes.
(3) About 80% ruptured membranes.

14 hours after insemination:

(2) About 30% ruptured membranes. The ciliation was still very weak.
(3) 90% hatched larvae. The larvae showed higher mobility than in the control.

14 hours 55 minutes after insemination:

(2) 80% with ruptured membranes. Low mobility.
(3) 100% hatched larvae.

16 hours 20 minutes after insemination:

(2) 90% hatched larvae swimming near the bottom with low mobility.
(3) The primary mesenchyme was well developed in (3) but was not present in the control or in (2).

meres. The position of the micromeres, squeezed in between the macromeres, may also facilitate intercellular contacts between these two types of cells.

As pointed out previously, lithium interferes with the rate of cleavage, and

TABLE III

Paracentrotus lividus, *eggs from one female. Temperature 18° C. (1) Control. The eggs were inseminated with 10^6 spermatozoa per ml. Eggs from the control were transferred into 0.05 M LiCl after: (2) 5 minutes; (3) 215 minutes*

120 minutes after insemination:	
	(1) 39% uncleaved, 61% 2-cell
	(2) 94% uncleaved, 6% 2-cell
140 minutes after insemination:	
	(1) 1% uncleaved, 99% 2-cell
	(2) 52% uncleaved, 48% 2-cell
165 minutes after insemination:	
	(1) 78% 2-cell, 22% 4-cell
	(2) 5% uncleaved, 95% 2-cell
200 minutes after insemination:	
	(1) 27% 2-cell, 70% 4-cell, 3% 8-cell
	(2) 87% 2-cell, 13% 4-cell
230 minutes after insemination:	
	(1) 64% 4-cell, 33% 8-cell, 3% 16-cell
	(2) 48% 2-cell, 48% 4-cell, 4% 8-cell
	(3) 65% 4-cell, 33% 8-cell, 2% 16-cell
260 minutes after insemination:	
	(1) 14% 4-cell, 68% 8-cell, 18% 16-cell
	(2) 15% 2-cell, 74% 4-cell, 11% 8-cell
	(3) 3% 2-cell, 20% 4-cell, 66% 8-cell, 11% 16-cell
290 minutes after insemination:	
	(1) 56% 8-cell, 44% 16-cell
	(2) 18% 2-cell, 42% 4-cell, 33% 8-cell, *7% 12-cell*
	(3) 3% 4-cell, 56% 8-cell, *2% 12-cell*, 39% 16-cell
320 minutes after insemination:	
	(1) 26% 8-cell, 71% 16-cell, 3% 32-cell
	(2) 15% 2-cell, 29% 4-cell, 10% 8-cell, *40% 12-cell*, 6% 16-cell
	(3) 2% 4-cell, 40% 8-cell, *5% 12-cell*, 53% 16-cell
365 minutes after insemination:	
	(1) 6% 8-cell, 62% 16-cell, 32% 32-cell
	(2) 2% 2-cell, 2% 4-cell, 6% 8-cell, *28% 12-cell*, 62% 16-cell
	(3) 7% 8-cell, *17% 12-cell*, 62% 16-cell, 14% 32-cell

within the larvae there appears to be a certain gradation in response to lithium; the different morphological regions of the embryo seem to be differently affected.

When the larvae have reached the 8-cell stage, and the process of cell division begins, which leads to the 16-cell stage, some of the embryos formed intermediate 12-cell stages (italicized in Table III) instead of the normal 16-cell stages. The cleavages in the four animal cells of the larva became temporarily inhibited when influenced by lithium, whereas the cleavages of the vegetal cells were not arrested to the same extent. The blocking of the cleavages in the animal region of the larvae is incomplete and temporary. As a consequence of the delayed formation of the presumptive mesomeres, the balance between the animal and the vegetal halves of the embryo becomes disturbed. The delayed cleavages in the animal half are also evident at the formation of the 32-, 64- and 128-cell stages, when larvae with reduced numbers of animal cells occur frequently. A typical experiment is shown in Table III.

Discussion

The results obtained in the present investigation indicate that the effects, which are recorded on cleavage after treatment with IBA and lithium, are correlated with the "animalization" and "vegetalization" observed during the ensuing development of the larvae. The enhanced or delayed rate of cleavage is not necessarily the direct cause of the animalizing or vegetalizing effect, but, in the author's opinion, the alteration in the rate of cleavage reflects the primary changes in the cells of the young larva, which result in the secondary events that occur during the later development.

The present observations indicate that the nuclei and chromosomes are affected by lithium, which interferes also with cell division. It has been shown that lithium ions cause a deficiency in the nucleoprotein synthesis in *Xenopus* embryos (Thomason, 1957), and this finding has probably some bearing on the present results.

The disruptive action of lithium on the cell contacts appears to be of special importance, because this is likely to render difficult the exchange of metabolites within the embryo. A phenomenon, which cannot be fully explained at present, is the high proportion of 12-cell stages (instead of 16-cell stages) observed after treatment with lithium. The fact that the reduced number of cells is dependent upon temporarily inhibited cleavages in the presumptive mesomeres indicates that the equilibrium within the larva is disturbed, and that the vegetal part of the embryo obtains a certain lead in development over the animal part. It may also indicate that the animal cells are more sensitive than the vegetal to exposure to lithium. The cell divisions are not equal, however (*cf.* Hörstadius, 1935), in the four animal and the four vegetal cells of the 8-cell stage, and consequently, they are not directly comparable. This indicates that the apparent differences in sensitivity to lithium may as readily be ascribed to the different orientation of the mitotic spindles in the animal and the vegetal cells, which may *per se* dispose the cells to respond differently to lithium. As has been previously mentioned, a similar effect of lithium was observed when the larvae were treated at the 16- to 32-cell stage, at the 32- to 64-cell stage, and at the 64- to 128-cell stage; larvae with less than the normal number of cells were also frequently encountered here.

Whether this disturbance of the cleavage pattern is to be attributed to a real difference in sensitivity between the animal and the vegetal cells of the embryo may, at present, be left an open question. At the cleavage stages between 16 and 128 cells, the cells of the animal and the vegetal halves are rather unequal. These cells have not the same surface areas, and the ratio, surface area/volume, is also different in the animal half from that in the vegetal. If we assume that the uptake and the action of lithium are correlated with the surface area exposed to the active ions, it may be justifiable to conclude that the deleterious effect on the animal part of the larva is visible on account of the larger surface area exposed to lithium. However, if there is any graded response to lithium in the different parts of the embryo, it is likely to be of a temporary nature. The elaborate experiments carried out by Lindahl and Holter (1940) point in the same direction. Their results showed that isolated animal and vegetal halves have the same oxygen consumption. Furthermore, it was demonstrated that treatment with lithium evokes the same inhibition of respiration in both animal and vegetal cells (Lindahl and Holter, 1940).

It is evident that lithium causes a general slowing down of early development. Lindahl (1936, 1941) showed that the oxygen consumption of larvae reared in the presence of lithium is considerably lower than that for larvae which develop in pure sea water; this is in agreement with the results reported here. The lithium effect becomes especially marked at the time of hatching, when the ciliation of the larvae treated is either reduced or entirely lacking. In some experiments, hatching took place before any ciliation of the larvae had developed, which may indicate that the production of the hatching enzyme (*cf.* Lundblad, 1954) is less affected by lithium than are the processes leading to ciliation.

The results obtained with IBA show that the action of this substance enhances cleavage. The ciliation of the larva is extremely well developed, and the rotatory movements inside the membrane often begin earlier in a treated batch of larvae than in the corresponding control. The actual rupture of the membrane is, however, delayed or even prevented. It was previously shown (Bäckström, 1955) that IBA does not interfere with the respiration of the larva up to the hatching stage, which is in accordance with the present results. IBA has not, as yet, been observed to cause any unbalanced increase in the cleavage of, for example, the animal half of the larva, but all cells within the embryo seem to be subject to the same effect that promotes cleavage. Though a number of substances and treatments have been found to induce animalization of the sea urchin larva, no common denominator for their physiological action has so far been discovered. It is therefore of interest to note that trypsin, which also induces an animalizing effect (Hörstadius, 1949, 1953), has recently been shown to bring about an accelerated rate of cleavage comparable with that resulting from treatment with IBA (Hagström and Lönning, 1962).

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SUMMARY

Under varied experimental conditions young sea urchin larvae were subjected to the action of solutions of o-iodosobenzoic acid and LiCl in sea water. Cleavage and early development were found to be advanced by IBA, and retarded by lithium. The fusion of the pronuclei was strongly inhibited by lithium, which tends also to separate the blastomeres. The rate of fertilization was not appreciably influenced by either IBA or lithium in the concentrations tested.

LITERATURE CITED

BÄCKSTRÖM, S., 1953. Studies on the animalizing action of iodosobenzoic acid in the sea urchin development. *Arkiv Zool.,* **4**: 485–491.

BÄCKSTRÖM, S., 1955. The effect of iodosobenzoic acid on the respiration of the developing sea urchin egg. *Arkiv Zool.,* **7**: 573–578.

GUSTAFSON, T., 1954. Enzymatic aspects of embryonic differentiation. *Intern. Rev. Cytol.,* **3**: 277–327.

HAGSTRÖM, B., AND B. HAGSTRÖM, 1954. A method of determining the fertilization rate in sea urchins. *Exp. Cell Research,* **6**: 479–484.

HAGSTRÖM, B. E., AND B. HAGSTRÖM, 1959. The effect of decreased and increased temperature on fertilization. *Exp. Cell Research,* **16**: 174–183.

HAGSTRÖM, B. E., AND S. LÖNNING, 1962. The effect of trypsin on the early development of the sea urchin egg. *Arkiv Zool.* in press.

HERBST, C., 1892. Experimentelle Untersuchungen über den Einfluss der veränderten chemischen Zusammensetzung des umgebenden Mediums auf die Entwicklung der Tiere. *Zeitschr. wiss. Zool.,* **55**: 446–518.

HÖRSTADIUS, S., 1935. Über die Determination im Verlaufe der Eiachse bei Seeigeln. *Pubbl. Staz. Zool. Napoli,* **14**: 251–429.

HÖRSTADIUS, S., 1939. The mechanics of sea urchin development, studied by operative methods. *Biol. Rev.,* **14**: 132–179.

HÖRSTADIUS, S., 1949. Experimental researches on the developmental physiology of the sea urchin. *Pubbl. Staz. Zool. Napoli,* **21**: Suppl. 131–172.

HÖRSTADIUS, S., 1953. Vegetalization of the sea-urchin egg by dinitrophenol and animalization by trypsin and ficin. *J. Embryol. Exp. Morph.,* **1**: 327–348.

LINDAHL, P. E., 1936. Zur Kenntnis der physiologischen Grundlagen der Determination im Seeigelkeim. *Acta Zool. (Stockholm),* **17**: 179–365.

LINDAHL, P. E., 1941. Physiologische Probleme der Entwicklung und Formbildung des Seeigelkeimes. *Naturwiss.,* **29**: 673–685.

LINDAHL, P. E., AND H. HOLTER, 1940. Beiträge zur enzymatischen Histochemie. Die Atmung animaler und vegetativer Keimhälften von *Paracentrotus lividus. C. R. Trav. Lab. Carlsberg, Sér. Chim.,* **23**: 257–288.

LUNDBLAD, G., 1954. Proteolytic activity in sea urchin gametes IV. Further investigations of the proteolytic enzymes of the egg. *Arkiv Kemi,* **7**: 127–157.

RUNNSTRÖM, J., 1954. Die Analyse der primären Differenzierungsvorgänge im Seeigelkeim. *Verhandl. deut. Zool. Ges. Tübingen,* **(3)**: 32–68.

RUNNSTRÖM, J., AND G. KRISZAT, 1952a. Animalizing action of iodosobenzoic acid in the sea urchin development. *Exp. Cell Research,* **3**: 497–499.

RUNNSTRÖM, J., AND G. KRISZAT, 1952b. The action of SH-reagents on the activation process in the sea urchin egg. *Arkiv Zool.,* **4**: 165–185.

THOMASON, D., 1957. Effect of lithium ions on the penetration of phosphate into *Xenopus* embryos. *Nature,* **179**: 823.

VON UBISCH, L., 1953. Entwicklungsprobleme. Gustav Fischer Verlag, Jena.

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OOGENESIS AND RADIOSENSITIVITY IN COCHLIOMYIA HOMINIVORAX (DIPTERA: CALLIPHORIDAE)

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Ionizing radiation and some chemicals are known to render insects sterile. Sterility may be achieved in two ways, by a treatment that inhibits the formation or development of mature sperm or ova (fecundity), or by a treatment that does not deter the production of these cells *per se,* but induces dominant lethal changes in their hereditary material, rendering them incapable of sustaining embryonic growth or causing death in the post-embryonic stages (fertility). The end result—sterility—is the same, but the processes involved in producing it are quite different. Many studies have investigated the reproductive system of both males and females and their differences in radiosensitivity with respect to the induction of dominant lethals, but inhibition of the growth of the reproductive organs has not been so widely studied.

In many species, growth of the ovaries is difficult to measure, especially in organisms that produce only a few mature gametes at a time. In some insects, however, the females produce a large number of gametes synchronously (egg masses). When many cells are undergoing growth and maturation simultaneously in many ovarioles, the reproductive organs attain considerable size at sexual maturity and the effect of a treatment can be easily measured.

Some aspects of the development of the female reproductive system in the screwworm fly, *Cochliomyia hominivorax* (Cqrl.), have been reported, in connection with work on radiation-induced dominant lethal mutations in developing oocytes (LaChance and Leverich, 1962). The present studies are concerned with the inhibition of ovarian growth by radiation, and with the morphological and cytological changes that lead to sterility characterized by infecundity. Particular emphasis is given here to the early growth stages, which are especially radiosensitive, and to the comparative radiosensitivity of the stages in oogenesis.

MATERIALS AND METHODS

Cochliomyia hominivorax is an obligate parasite of warm-blooded animals. The biology and laboratory-rearing procedures for this insect have been discussed in several publications (see Bushland, 1960). The insects used in the experiments reported here were of the laboratory strain, reared on artificial medium. In this rearing technique, the larvae pass through the three instars in the artificial medium and pupate in sand. The length of the pupal period is approximately 7–7½ days at 80° F. The adults were reared at 80° F. and fed honey and water, a diet that supports life and ovarian growth very well. Laboratory-reared adults mate when approximately two days old. The females are gravid at 5–6 days of age, and will produce an egg mass of more than 200 eggs when oviposition is induced by presenting the females with a small piece of lean meat and keeping them at a temperature of 90–96° F. for a few hours. A second egg mass may be produced 3–5 days later.

The radiation exposures were performed in air in a Co^{60} radiation source (Jefferson, 1960) at a dose rate of 642–683 roentgens per minute. Calibration of the unit was accurate to ±6%.

In the studies of ovarian growth, both ovaries were dissected from individual females; 30 females were examined for each treatment group. The dimensions of each ovary were measured with an ocular micrometer in the eyepiece of a dissecting microscope (17 ×). One measurement of height and two of diameter (at right angles to each other) were made. Estimation of ovarian volume is somewhat difficult, since the organ does not resemble any geometric form precisely. The several formulae used to calculate the size of a rounded geometric object have certain values that are constant. However, since the measurements made in these experiments were not intended to establish an exact volume but to reduce the observations to a single number that could serve as a basis for comparison between groups, the volume of the ovaries was approximated by multiplying the three measurements and correcting for the magnification.

In the studies of ovarian growth in *Cochliomyia,* temperature was repeatedly observed to affect size: Females of equal age reared at 80° F. had larger ovaries than those reared at 73° F., and sexual maturity was reached in 5 days at the higher temperature whereas 7 days was required at the lower temperature. For this reason, rearing temperatures in these experiments were all closely controlled at 80° F.

For the cytological studies, whole mounts of ovarioles were made from females of various ages and stained on a microscope slide with the Feulgen procedure. In all studies, corroborative evidence was gained by examining freshly prepared mounts in insect saline with a Zeiss phase-contrast microscope at 1600 ×. The staining procedure causes considerable shrinkage of the ovarioles, so those examined in wet mounts were always somewhat larger than the Feulgen-stained preparations.

RESULTS AND DISCUSSION OF INDIVIDUAL EXPERIMENTS

1. *Growth of the ovaries in normal females*

Data on the measurements of the ovaries in normal females of various ages are summarized in Table I. Newly emerged females had immature ovaries with volumes ranging from 0.068 to 0.254 mm^3. The size of the ovary doubled between the first and second day of adult life and increased by more than five-fold between the second and third day of adult life. Growth then continued somewhat more slowly until the mature ovaries had attained a size of more than 7 mm^3. Thus, from emergence until sexual maturity, the size of the ovary increased approximately 60-fold.

The growth of the ovary is the manifestation of the growth processes occurring synchronously in the more than 100 ovarioles that comprise each ovary. A cytological study of the ovarioles was conducted to determine the sequence of events in normal oogenesis, so that a basis could be established for evaluating the effects of

TABLE I

Growth of ovaries in normal Cochliomyia hominivorax. (Each number represents the mean from 30 females ± the standard error of the mean.)

Age of female (days ± 2 hours)	Mean volume of ovaries ± $s_{\bar{x}}$ (mm.^{3})	
	Right	Left
0–2 hours	0.1423 ± 0.0225	0.1273 ± 0.0015
1	0.2022 ± 0.0095	0.2002 ± 0.0023
2	0.4094 ± 0.0470	0.3952 ± 0.0362
3	2.1064 ± 0.204	2.2446 ± 0.234
4	5.3841 ± 0.328	5.0761 ± 0.347
5	7.3885 ± 0.274	7.3921 ± 0.274

radiation or other damaging agents on the growth of the reproductive organs and on other factors affecting female sterility.

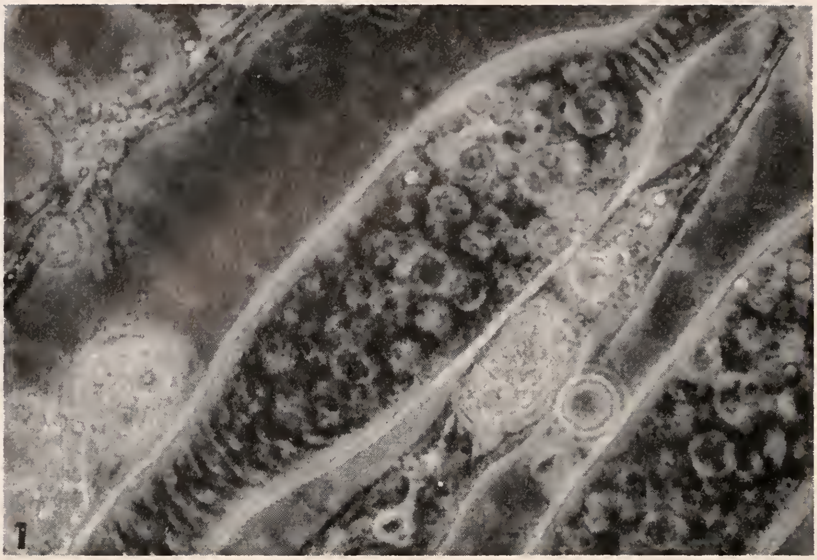
2. *Cytology of normal ovarioles*

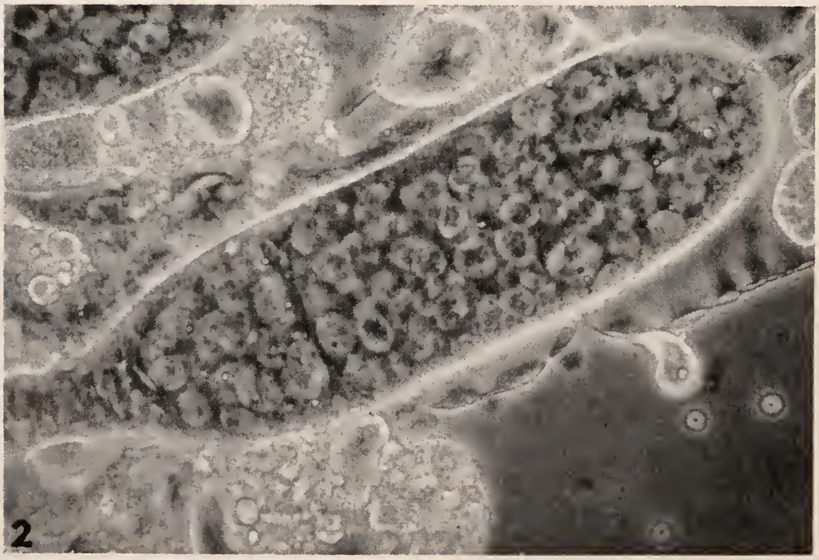
The ovary in *Cochliomyia* consists of 100–150 ovarioles, each of which is enclosed in an epithelial sheath and produces one mature egg to be deposited in each egg mass. Oocyte development is synchronous in all ovarioles. Scale drawings showing the normal developmental sequence in an ovariole are presented in Figures 8 and 9. The cytological studies showed the following pattern of oogenesis correlated with the age of the female.

Pupae 4 and 5 days old. The ovary is small and immature. Each ovariole is composed of a germarium filled with oogonial cells (Fig. 8A and Fig. 1). These cells are mitotically active, and nests of four or eight cells in division are occasionally seen in the anterior and middle portion of the germarium. There is no evidence of nurse cell formation. Examination of ovarioles from 4- and 5-day-old pupae, in Feulgen-stained preparations or in phase-contrast studies of fresh whole mounts in saline, did not show any differentiation of the germarial contents.

Pupae 6 days old. The ovariole is still composed of a single germarium, surrounded by an envelope of epithelial cells (Fig. 8B and Fig. 2). Mitotic divisions are now relatively rare. In some of the ovarioles the nuclei in the posterior portion of the germarium have enlarged very slightly, and the chromatin material has become slightly more diffuse. In some ovarioles a faint line of separation is seen between the anterior and posterior portion of the germarium (Fig. 2); this is the first indication of the first egg chamber being formed.

Pupae 7 days old. A cyst has formed in the posterior portion of each ovariole, indicating formation of the first egg chamber. In some ovarioles dissected from pupae almost ready to emerge, an indentation has formed that separates and "pinches off" the first egg chamber from the germarium (Figs. 3 and 8C). This newly formed chamber contains 16 cells, 15 of which are large and distinct nurse cells and the other, the smaller oocyte. The 15 nurse cells have not differentiated noticeably, except that the nuclei contain diffuse chromatin material. The germarium occasionally contains a smaller second cyst, in the posterior region, that is destined to become the second egg chamber (Fig. 3). This structure was visible in phase contrast; in Feulgen-stained preparations, changes in nuclear morphology were not observable in the remaining germarial contents.





FIGURES 1–2. Phase contrast, unfixed tissue in saline, 640 ×. Figure 1. Ovariole from untreated 5-day-old pupa. Figure 2. Ovariole from untreated 6-day-old pupa; note furrow forming in posterior region of germarium.

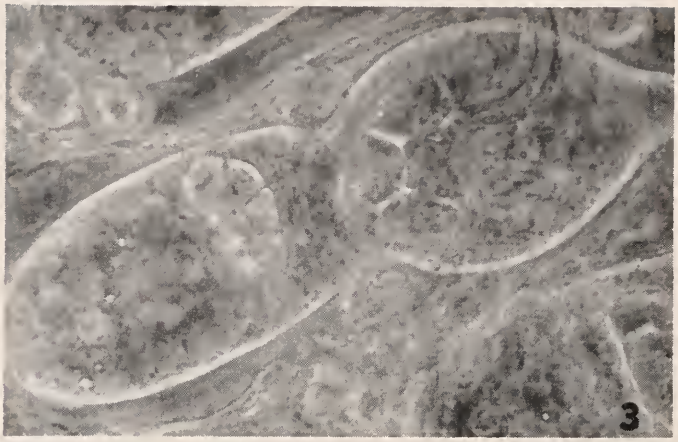
Presumably the 16 cells of an egg chamber derive from four consecutive divisions of an oogonial cell, maintain a positional relationship to one another, and are subsequently incorporated into an egg chamber or cyst, but in *Cochliomyia* there was no indication that these cells are enclosed in a cyst until the pupae are 6½–7 days old. Very likely the daughter cells are interconnected by fine protoplasmic strands, but these were not demonstrable with the techniques used in these studies. Hirschler (1955) has presented an excellent study of the early positional relationship of the cells that will subsequently develop into an egg-nurse cell complex in insects. He states (p. 78), "After a given number of divisions, the egg remains united to the conceivably greatest number of nurse-cells by means of cell bridges functioning as nutritive ducts. As far as nutrition is concerned, the egg lies in the best possible situation." The studies of King (1960) also indicate that these daughter cells remain connected by intercellular cytoplasmic bridges and are subsequently incorporated into an egg chamber. Fawcett *et al.* (1959) have studied the intercellular bridges that exist between daughter cells derived from an original parent cell in the spermatids of many species, and suggest that protoplasmic continuity between these cells is the basis for their synchronous development. It is quite possible that the synchronous development of ovarian nurse cells is also related to their intercellular connections.

Adult females 0–4 hours old. The 15 nurse cells in the first egg chamber are greatly enlarged (Fig. 8D). The chromosomes in the nurse cell nuclei are thickened and stain deeply with Feulgen stain (Fig. 4A and 4B). Formation of polytene chromosomes in ovarian nurse cells is quite common in Diptera (Stalker, 1954; Bier, 1960). In *Cochliomyia* the reduplicated chromonemal strands remain in close enough association to appear as greatly thickened chromosomes (Fig. 4A), but not so close as to give a very definite banded appearance as in some other species (Stalker, 1954; Bier, 1960).

Adult females 4–24 hours old. The major changes in the first egg chamber take place in the nurse cell nuclei, which continue to enlarge progressively, due to the replication of chromosomal material. This process results in the formation of polytene chromosomes. Chromonemal reproduction in the nurse cells is followed by a complete separation of the reproduced elements. The separation does not involve a regular dicentric anaphase, but merely a complete dissociation of the numerous strands or a general "falling apart," resulting in a mass of chromatin fibrils that completely fill the nuclear volume (Fig. 5). The entire process takes place within an intact nuclear membrane and may, according to the terminology of Lorz (1947) and others (see Painter and Reindorp, 1939; Painter, 1959), be termed endomitosis. This endomitotic process has been described in great detail by Painter and Reindorp (1939) and King (1960) for *Drosophila,* and by Bier (1960) for *Calliphora.* In *Cochliomyia* it is very similar, except that it takes place simultaneously in hundreds of ovarioles. Jacob and Sirlin (1959) have reported that in *Drosophila* the most posterior nurse cells undergo one more duplication than the more anterior cells, and become larger and more active. The posterior nurse cells in *Cochliomyia* are also distinctly larger (Fig. 5), and very likely undergo more replications of chromosomal material than do the anterior nurse cell nuclei.

In these studies, only rarely were the polytene chromosomes of the nurse cells observed to dissociate before the females were 8 hours old. In ovarioles from females 8–16 hours old, the thickened polytene chromosomes had dissociated into Feulgen-positive chromatin threads in approximately half of the nurse cells.

Adult females 24 hours old. The endomitotic process is completed during the first day of adult life. After 24 hours all the nurse cell nuclei are filled with loosely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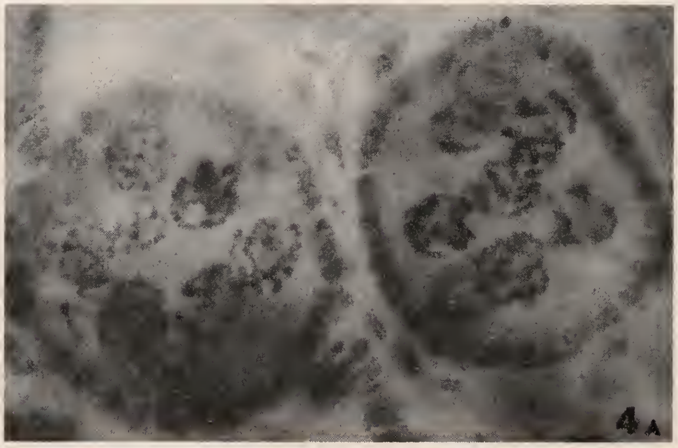
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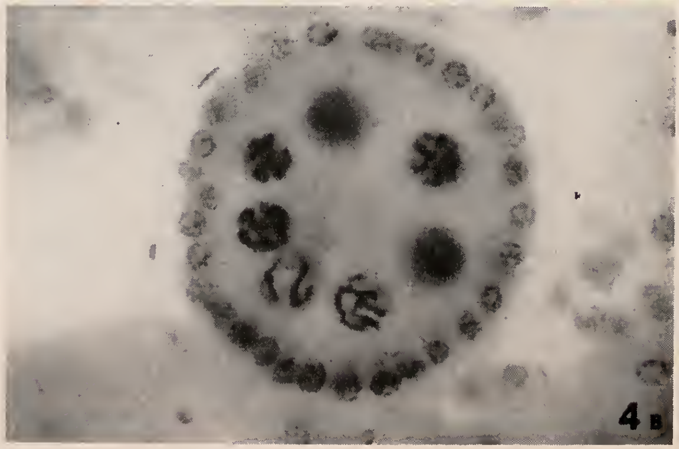
4B

associated Feulgen-positive chromatin threads (Fig. 5). The nurse cells have now enlarged to their greatest size. The ovariole consists of an enlarged first egg chamber, the second chamber is beginning to form in some ovarioles, and the germarium is filled with oogonial cells (Fig. 9A). In contrast, the oocyte nucleus in newly emerged females is smaller and stains very lightly with Feulgen stain throughout the first day. In these studies the nucleolus did not stain, but it was clearly visible in phase-contrast examination.

Adult females 48 hours old. The first egg chamber has enlarged considerably, so that the nurse cells are not so closely packed, but more dispersed in the chamber. The oocyte nucleus is very small and spherical, and is located in the posterior region of the egg chamber. The second egg chamber has now clearly formed in all ovarioles. The nurse cells in the first egg chamber are extruding a fine granular material which stains faintly with Feulgen; this material is beginning to fill the egg chamber.

Adult females 3 days old. Between the second and third day after emergence, the first egg chamber in each ovariole more than doubles in size (see Table I). The nurse cells are localized at the anterior end of the chamber (Fig. 9B and C). The oocyte has grown noticeably and is beginning to elongate; the oocyte nucleus is located in the ooplasm very near the nurse cells and is in prophase I of the first meiotic division (LaChance and Leverich, 1962). The second egg chamber has also enlarged and contains nurse cells undergoing endomitotic replications. Some of the nurse cells in the second egg chamber have completed the process of endomitotic growth and dissociated chromosomal material is beginning to fill the nucleus.

Adult females 4 days old. The mature ovum is now almost fully formed. The nurse cells, when present, stain much more deeply and appear to darken and disintegrate. Most nurse cells in the upper portion of the egg follicle have disappeared (Fig. 9D). The disappearance of the nurse cells from the first egg chamber is correlated with the passage of the oocyte nucleus from prophase I to metaphase I of the first meiotic division (LaChance and Leverich, 1962).

Adult females 5 days old. Each ovariole contains a mature ovum ready for oviposition (Fig. 7a). The nurse cells have completely disappeared and the oocyte nucleus is now in anaphase I of the first meiotic division (LaChance and Leverich, 1962). All nurse cells in the second egg chamber have completed endomitotic growth and contain nuclei filled with fine Feulgen-positive threads existing in great multiplicity. A very small third egg chamber has just formed from the germarium.

Thus, at the time when the first egg mass is deposited, each ovariole contains a well developed second egg chamber, which will then repeat the process described above and form a mature ovum to be deposited in the second egg mass. At 5 days of age, growth in the ovarioles is arrested until deposition of the first egg mass; cytological preparations of ovarioles from females 7–9 days of age that have not oviposited closely resemble those of females 5 days of age.

FIGURE 3. Phase contrast, unfixed tissue in saline, 640 ×. Ovariole from untreated pupa 7–7½ days old; first egg chamber well formed (upper right), germarium containing a second cyst (lower left).

FIGURE 4. Feulgen-stained whole mounts, 800 ×. Egg chambers in ovarioles from untreated adults 0–4 hours old. (A) Two adjoining egg chambers, showing polytene nurse cell chromosomes (lower focal level). (B) One egg chamber, showing polytene nurse cell chromosomes (upper focal level).

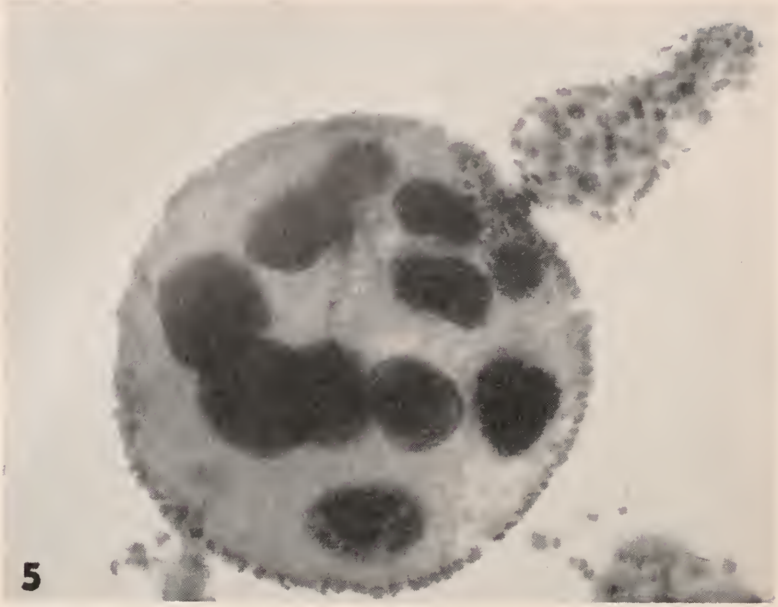

FIGURE 5. Feulgen-stained whole mount, 512 ×. Ovariole from untreated 28-hour-old adult; note 15 nurse cell nuclei containing dissociated chromatin fibrils, oocyte nucleus very faintly stained (lower left of first egg chamber).

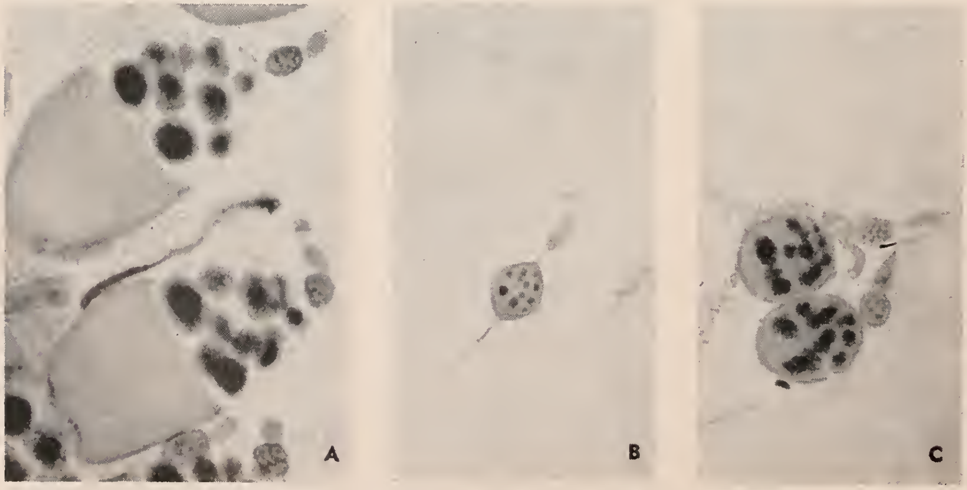

FIGURE 6. Feulgen-stained whole mounts, 80 ×. Ovarioles from 2½-day-old adults. (A) Control; shows large first egg chamber with 15 enlarged nurse cells, second egg chamber, and germarium. (B) Treated with 2000 r as 5-day-old pupa; note absence of second egg chamber. (C) Treated with 2000 r as adult 0–4 hours old.

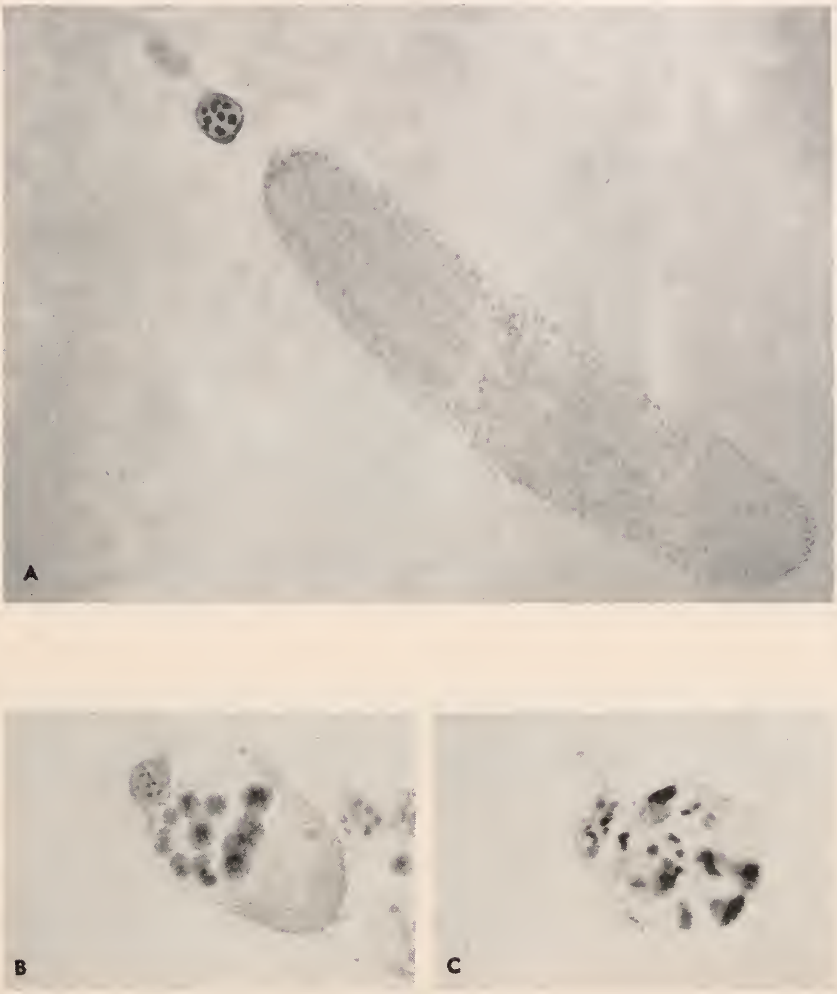

FIGURE 7. Feulgen-stained whole mounts, 80 ×. Ovarioles from 4½-day-old adults. (A) Control. (B) Treated with 4000 r as 5-day-old pupa; note typical undeveloped first and second egg chambers and atrophied germarium. (C) Treated with 4000 r as adult 0–4 hours old; note typical malformed egg chambers and germarium.

3. *Effects of irradiation on ovarian growth*

With the data obtained on ovarian measurements and the cytological studies of ovarioles from normal females of various ages, it was possible to study the effects of gamma radiation treatments, and to determine which ages presented the reproductive system in stages of highest radiosensitivity or resistance. By observing the inhibition of ovarian growth induced in females of various ages by a given dose of radiation, it was possible to compare the sensitivity of oogonial cells, of egg chambers in which the nurse cells were undergoing endomitotic growth, and of

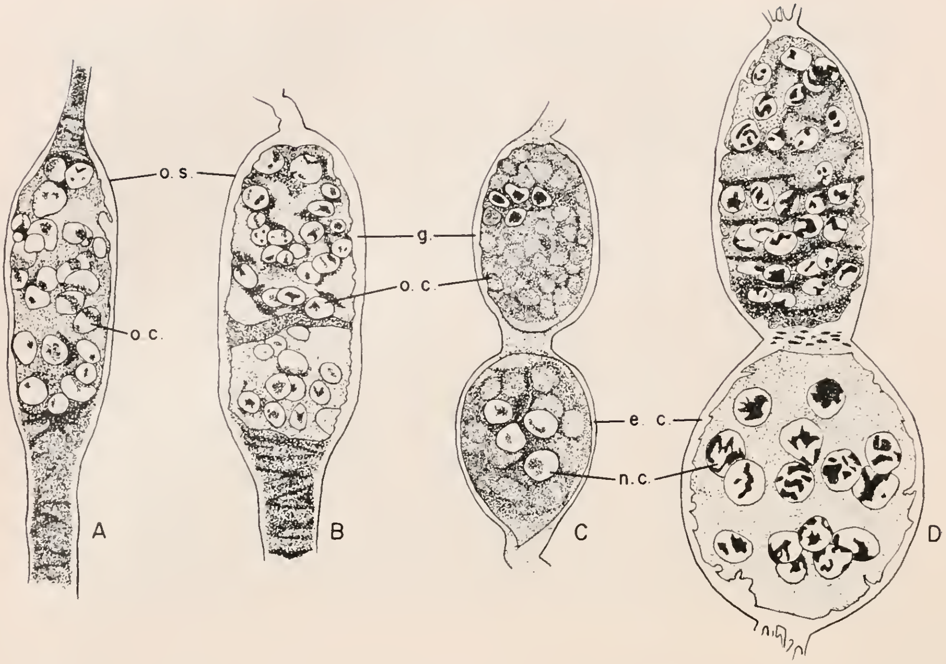

Figure 8. Normal ovarian development in *Cochliomyia hominivorax*. Each figure represents a single ovariole; drawings prepared from unfixed whole mounts examined in phase contrast (enlarged 400 ×). (A) From pupa 4½–5 days old. (B) From pupa 6 days old. (C) From pupa 7½–8 days old. (D) From adult 0–4 hours old. (o.s. = ovariole sheath; g. = germarium; e.c. = first egg chamber; o.c. = oogonial cells; n.c. = nurse cells.)

older egg chambers in which endomitosis was completed but vitellogenesis was in progress. The results of these comparisons are presented in Table II.

The early experiments of Bushland and Hopkins (1953) showed that larvae and young pupae were severely injured by irradiation and emergence was reduced. However, 6-day-old pupae could be sterilized by a dose of 5000 r. When pupae were treated at this level, most of the adult females did not produce eggs and the few that did produced a very few eggs, which did not hatch; the adult males produced motile sperm that contained dominant lethals. In further work on 6-day-old pupae (Baumhover *et al.*, 1955), it was found that a dose of 7500 r was required to prevent egg production completely. The studies in Table II show that the process of ovarian growth can be inhibited at other times in the life cycle with smaller doses of radiation. Thus, although between 4000 and 5000 r were required to inhibit growth of the ovaries when 5-day-old pupae were irradiated, newly emerged females were much more radiosensitive: a dose of 2000 r reduced ovarian growth by half, and almost complete inhibition of ovarian growth was obtained with 4000 r. However, when two-day-old females were given even higher doses (up to 8000 r), ovarian growth and egg production were not greatly affected. It should be noted that, although irradiation of older females does not greatly inhibit

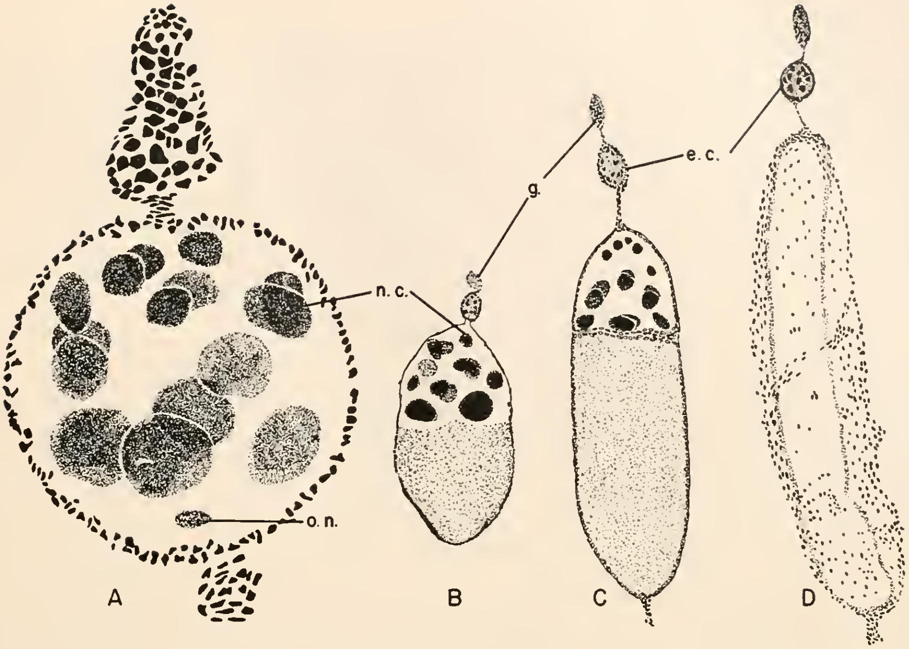

FIGURE 9. Normal ovarian development in *Cochliomyia hominivorax*. Each figure represents a single ovariole; drawings prepared from stained material. (A) From adult 28 hours old (384 ×). (B) From adult 2½ days old (60 ×). (C) From adult 3½ days old (60 ×). (D) From adult 4 days old (60 ×). (n.c. = nurse cells; o.n. = oocyte nucleus; e.c. = second egg chamber; g = germarium.)

ovarian growth, sterility can still be achieved by inducing dominant lethal changes in the formed or forming oocytes so that, even though eggs are produced, embryos will not develop (LaChance and Leverich, 1962).

TABLE II

Effects of gamma radiation on ovarian growth in Cochliomyia hominivorax. (Each number represents the mean from 30 5-day-old females ± the standard error of the mean.)

Radiation dose (r)	Mean volume of ovaries ± $s_{\bar{x}}$ (mm.^{3}) from females treated as:					
	5-day-old pupae		Adults 0–4 hours old		Adults 48–52 hours old	
	Right	Left	Right	Left	Right	Left
Control	8.1410 ± 0.277	8.3247 ± 0.375	8.3400 ± 0.350	8.3529 ± 0.381	8.3400 ± 0.350	8.3529 ± 0.381
2000	8.1102 ± 0.264	8.1696 ± 0.276	4.4966 ± 0.403	4.3916 ± 0.426	7.4108 ± 0.356	7.3072 ± 0.344
4000	3.6555 ± 0.278	3.6035 ± 0.251	1.7251 ± 0.142	1.6723 ± 0.104	6.4428 ± 0.390	6.7258 ± 0.421
5000	0.9583 ± 0.120	0.9077 ± 0.112	1.2828 ± 0.074	1.2200 ± 0.078	7.1426 ± 0.332	7.3094 ± 0.348
6000	0.4327 ± 0.033	0.4511 ± 0.040	0.8968 ± 0.069	0.8867 ± 0.068	6.5205 ± 0.375	6.4219 ± 0.424
8000	0.2503 ± 0.018	0.2646 ± 0.018	1.0820 ± 0.275*	1.1390 ± 0.293*	7.0346 ± 0.419	6.9750 ± 0.426

* Mean from 18 females only.

4. *Cytology of the irradiated ovary*

To investigate the cytopathological changes related to the inhibition of ovarian growth after irradiation, a further series of experiments was conducted. Females were irradiated with 2000 or 4000 r, either as 5-day-old pupae or as newly emerged adults 0–4 hours old. The ovaries were dissected from the females at 2½, 4½, and 5½ days after emergence and the ovarioles teased apart, fixed, and stained. The slides were made permanent and were later examined microscopically for the degree of development, presence or absence of nurse cells, presence or absence of second egg chambers, and staining differences in the nuclear components, as compared with a suitable set of controls. The slides were coded before examination to prevent bias in recorded observation. The results are presented in Table III.

Occasionally in the dissecting and staining procedure, some portion of the ovariole was torn or lost. This condition occurred most frequently in the area between the first and second egg chambers, which are connected by a very thin sheath. Therefore, at times only the first egg chamber could be observed for cytological changes after a treatment, and the condition of the rest of the ovariole remained unknown. For this reason, there are more first egg chambers recorded in Table III than second egg chambers or germaria. In treatment group A, for example, 150 ovarioles were examined, but of these only 114 included the second egg chamber, and 103 contained the complete ovariole including the germarium. In no instance was any component scored as absent if any possibility existed that it had been lost in dissection; when second egg chambers are recorded as absent, both the first egg chamber and the germarium were present within the ovariole sheath, and the second egg chamber had clearly failed to develop (Fig. 6B).

Altogether, 1406 ovarioles were examined. The amount of information originally collected has been summarized for presentation in Table III. In order to organize the data, it was necessary to separate the appearance of ovarioles and their contents into categories as follows:

Reduced normal indicates that the entire structure, including nurse cells, appeared normal in shape and degree of staining, but was distinctly smaller in size than controls of similar age. This term for the first egg chamber indicates that the nurse cells had begun to migrate toward one end of the follicle and that 15 nurse cells were present in nearly all instances. For the second egg chamber, all the structures were present but reduced in size. *Undeveloped* refers to the first egg chambers and indicates that the chambers were very small but usually contained 15 nurse cells. Normal development had been retarded so that, in comparison with controls of similar age, the ovarioles appeared much younger as well as smaller. The nurse cells in the chamber were scattered throughout the entire structure rather than tending to gather at the polar end (Fig. 6C). *Malformed* also refers to first egg chambers and is used to describe ovarioles from the older groups (4½ and 5½ days old when dissected) that were so badly stunted in size and development that they could not even be considered "undeveloped" (Fig. 7C). Many egg chambers were misshapen and contained pycnotic or degenerate nurse cells; the chambers were extremely small for their age and showed no signs of development.

Absent in reference to the second egg chamber indicates a complete lack of a recognizable object between the first egg chamber and the germarium, and a vacant space in the ovariole sheath (Fig. 6B). *Atrophied* means that a second egg

TABLE III

Cytopathology of the irradiated ovary in Cochliomyia hominivorax. Females treated with gamma radiation and the ovarioles dissected for study at indicated days post-treatment. See text for definition of column headings

Treatment					First egg chamber						Second egg chamber						Germarium			
Group	Dose (r)	Age treated	Age dissected (days)	Number observed	Reduced normal		Undeveloped		Malformed		Reduced normal		Absent		Atrophied		Reduced normal		Degenerate	
					No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
A	2000	5-day pupae	2½	150			150	100.0			78	68.4	36	31.6			103	100.0		
B	2000	5-day pupae	4½	120	105	87.5	15	12.5			7	87.5	1	12.5			6	100.0		
				Total 270	105	38.9	165	61.1			85	69.6	37	30.3			109	100.0		
C	2000	0–4-hour adults	2½	208	101	48.6	107	51.4			134	85.9	22	14.1			144	99.3	1	0.7
D	2000	0–4-hour adults	4½	79	25	31.6	9	11.4	45	57.0	33	89.2	4	10.8			25	78.1	7	21.9
E	2000	0–4-hour adults	5½	76	1	1.3			75	98.7	36	83.7	1	2.3	6	14.0	29	78.4	8	21.6
				Total 363	127	35.0	116	32.0	120	33.1	203	86.0	27	11.4	6	2.5	198	92.5	16	7.5
F	4000	5-day pupae	2½	102			102	100.0			28	50.9	27	49.1			45	100.0		
G	4000	5-day pupae	4½	66			66	100.0			3	14.3	18	85.7			9	60.0	6	40.0
				Total 168			168	100.0			31	40.8	45	59.2			54	90.0	6	10.0
H	4000	0–4-hour adults	2½	172			172	100.0			65	59.1	45	40.9			70	68.0	33	32.0
I	4000	0–4-hour adults	4½	146					146	100.0	70	85.4	7	8.5	5	6.1	58	87.9	8	12.1
J	4000	0–4-hour adults	5½	60	2	3.3	56	93.3	2	3.3	1	5.3	18	94.7			8	42.1	11	57.9
				Total 378	2	0.5	228	60.3	148	39.1	136	64.5	70	33.2	5	2.4	136	72.3	52	27.7

chamber was present but small and misshapen, and frequently with no nurse cells or very poorly defined ones.

A *reduced normal* condition for the germarium means the same as for the first and second egg chambers: The structure appeared normal but was much smaller in size than controls of the same age. A *degenerate* germarium indicates that there was evidence of the presence of a germarium but that it was so shrunken in appearance that it could not be considered merely reduced in size (Fig. 7B).

Controls for these experiments were untreated females dissected at 2½, 4½, and 5½ days after emergence. The appearance of normal ovarioles at various ages has been described in section 2, and photographs are given in Figures 6A and 7A.

Some cytological observations from these experiments could not be readily categorized for presentation in Table III, and are discussed in the following paragraphs. These comments are intended to supplement the data in Table III. The data in the table were based on comparisons of ovarioles from the control groups with those from treatment groups. *No* abnormalities were found in the controls, but in none of the treated females was completely normal development of an egg chamber ever observed, even at low, non-sterilizing doses of radiation. All ovarioles from treated females were distinctly retarded in growth even if nothing else appeared abnormal.

Five-day-old pupae treated with 2000 r (treatment groups A and B). Of 150 ovarioles dissected from females 2½ days after emergence (group A), all could be classified as undeveloped, and 10 of these were very small with tiny nurse cells. This pattern was interpreted as retardation of growth rather than cessation, for when ovarioles from similarly treated females 4½ days old were examined (group B), 87.5% had developed to a stage that would normally be found in control females two or three days old—that is, the nurse cells were very small but still evident and occupying half the volume of the first egg chamber. Of the 270 first egg chambers examined in the two groups, all had 15 nurse cells that were reduced in size. Undeveloped nurse cells could result in a much slower rate of vitellogenesis and thus account for the small or undeveloped egg chambers. The observations on second egg chambers indicated that these also formed at a much later time than in normal ovarioles. When ovarioles from 2½-day-old females were examined, 31.6% had failed to develop second egg chambers; of the ovarioles from 4½-day-old females, 87.5% had a second egg chamber but it was small for the age group. Apparently the process of ovogenesis in the contents of the ovarioles was retarded at least two days by this treatment. The most obvious trend was a slowing down of all processes involved in ovogenesis, but not a complete cessation of development.

Adults 0–4 hours old treated with 2000 r (treatment groups C, D, and E). Of the 208 ovarioles dissected at 2½ days (group C), all had 15 nurse cells that were normal in appearance but reduced in size. In the 79 ovarioles dissected from 4½-day-old females (group D), 45 had malformed ova; 34 had egg chambers that were reduced in size or undeveloped, and of these 26 had 15 nurse cells that were reduced in size, 4 had less than 15 nurse cells, and 4 had pycnotic or degenerate nurse cells numbering 14 or less. In the 76 ovarioles dissected from females at 5½ days (group E), 98.7% of the first egg chambers examined were malformed, and most of these lacked nurse cells or contained shrunken, degenerate nurse cells.

In these three treatment groups, 236 ovarioles were complete enough to permit observation of second egg chambers, but in only 203 of these were the second egg chambers developed. In group D, of 37 complete ovarioles examined, 4 were lacking a second egg chamber, 25 had formed a second chamber in which there were 15 small nurse cells, 7 had formed a chamber with definitely abnormal nurse cells, and one had a chamber with less than 15 nurse cells. Most germaria observed in the complete ovarioles were normal in appearance but reduced in size when compared with controls of similar age; 16 were completely degenerate and misshapen.

In general, the damage to the oocytes was greater after treatment of females 0–4 hours old with 2000 r than after a like treatment of 5-day-old pupae (see Table II). Instead of merely slowing down the process of ovogenesis and allowing the delayed production of some normal eggs, treatment of newly emerged females seemed to result in a preponderance of malformed eggs. Although the second egg chambers were usually developed to some extent (86%), their growth was reduced considerably and was occasionally followed by atrophy. In addition, degeneration of germaria was observed; this condition did not occur after treatment of 5-day-old pupae.

Five-day-old pupae treated with 4000 r (treatment groups F and G). The first egg chambers that developed following treatment with 4000 r grew only very slightly and then remained almost stationary in size. Of the 168 first egg chambers examined, none had progressed past the "undeveloped" stage. Of 66 first egg chambers dissected at 4½ days, 47 contained 15 nurse cells of reduced size, 17 contained from 2 to 14 small nurse cells of nearly normal appearance, and two contained pycnotic or degenerate nurse cells.

In these two treatment groups, 76 complete ovarioles were observed, but in only 31 of these were second egg chambers developed. Of the 55 complete ovarioles dissected at 2½ days, second egg chambers were absent in 27 ovarioles; second chambers had formed in 28 ovarioles but were extremely reduced in size, and in only 8 of these was it possible to observe 15 nurse cells. Of the 21 complete ovarioles dissected at 4½ days, 18 did not form second egg chambers, and of the remaining three that did, 15 nurse cells were observed in only one and these were reduced in size.

The general trend of ovarian development after treatment of 5-day-old pupae with 4000 r was a retardation of growth in the first egg chamber. There were no instances of the first egg chamber progressing to the point of formation of an ovum without nurse cells; growth was apparently arrested at an early stage of development. Second egg chambers had failed to develop in 60% of the complete ovarioles studied, and both reduction in growth rate and atrophy were observed in the germaria.

Adults 0–4 hours old treated with 4000 r (treatment groups H, I, and J). Of the 378 first egg chambers observed, 228 were undeveloped and 148 had developed into malformed and abnormal oocytes (Fig. 7C). Of 172 first egg chambers from females dissected at 2½ days (group H), all appeared undeveloped, with 15 nurse cells of reduced size. Of 146 first egg chambers from 4½-day-old females (group I), all were both undeveloped and malformed; 58 of the follicles still had 15 very small nurse cells, but in the remaining 88 the nurse cells had begun to degenerate and were difficult to count precisely (Fig. 7C). Of the 60 ovarioles from 5½-day-old females (group J), only two could be classified as normal in appearance but distinctly retarded in growth; 56 were still undeveloped, and two malformed. Of the first egg chambers from these 60 ovarioles, 35 contained approximately 15 nurse cells of reduced size and 25 contained pycnotic or degenerate nurse cells.

Of 211 complete ovarioles from these three groups, the second egg chamber had failed to develop in 70, in 5 it was atrophied, and in 136 it was very much reduced in size. There was a relatively high incidence (28%) of germaria being found in a degenerate state or absent altogether; in all other instances in which the germarium was observed, its size was reduced.

Previous work has shown that a treatment of 4000 r given to females 0–4 hours old results in complete sterility (LaChance and Leverich, 1962), whereas a similar treatment of 5-day-old pupae results in considerable damage to the reproductive tissues but does not fully sterilize the females (Bushland and Hopkins, 1953).

GENERAL DISCUSSION

It is tempting to compare the mode of action of the various agents that are known to cause sterility. Since *Cochliomyia hominivorax* is currently being tested with a number of chemosterilants, it is hoped that the present observations on the pattern of radiation-induced sterility in the female of this species will serve as a basis for future comparisons against the effects of chemical sterilization.

The present studies demonstrate that the effect of irradiation on the reproductive capability of female *Cochliomyia* is largely dependent on the stage of development of the ovarioles at the time the radiation treatment is administered. The time during which the egg chambers contain nurse cells undergoing endomitotic replications of chromosomal material (adults 0–4 hours old) was the most radiosensitive stage encountered in these studies. Irradiation during this period is much more likely to be followed by infecundity than when an equivalent dose of radiation is delivered before endomitosis begins (5-day-old pupae) or after it has been completed (24-hour-old adults) (see Table II).

The present studies corroborate the observations of Grosch and Sullivan (1954) that the period of endomitosis is especially vulnerable to damage by irradiation and that, following treatment at this stage, growth is hampered. There are several possible reasons why a group of nurse cells would present an especially vulnerable target during the endomitotic process. Although it is true that the polyploid nurse cells in very young females present a multiplicity of chromosomal targets as compared with the diploid oogonial cells in 5-day-old pupae, it is not likely that greater radiosensitivity is associated merely with the greater content of DNA in the nurse cells. The nurse cells in females 24 hours old certainly contain as much, or more, DNA as those of younger females, yet 24-hour-old females are so resistant to radiation treatments that even doses of 8000 r do not seriously hamper egg production. Rather, we favor the idea that the failure of the treated females to produce mature ova reflects an inability of the nurse cells to support normal vitellogenesis. If a radiation treatment is given before the endomitotic replication of nurse cell chromosomal material is completed, the process is likely to be arrested, and the result is the formation of nurse cells without the elevated complement of chromosomal material in the nuclei. King and Sang (1959) have suggested that vitellogenesis cannot proceed to completion without this elevated chromosomal complement. One of the most common features in the present cytological studies of irradiated ovarioles was nurse cells that were fairly normal in appearance but very much reduced in size.

The formation of immature egg chambers with abnormally small nurse cells could lead to such changes in the process of oogenesis as a slowing down of vitellogenesis or, often, a general cessation of growth at the point at which vitellogenesis would normally be most active and contribute most to further increases in the size of the oocyte. Occasionally the nurse cells degenerated before growth of the oocyte was complete, which resulted in the production of very few eggs or malformed eggs that did not hatch.

On the other hand, there remains the possibility that the oocyte nucleus changes in radiosensitivity during the various stages of oogenesis. Damage to the oocyte nucleus could conceivably affect the process of vitellogenesis by removing in some manner the stimulus for the nurse cells to produce yolk. There is some evidence that this factor operates in *Drosophila:* In studies of ovarian tumors in this species, King *et al.* (1961) observed that nurse cells will form without the presence of an oocyte, but that yolk is not produced without an oocyte nucleus in the chamber.

Ovarian tumors similar to those observed by King (1957) were not found in the present experiments with female *Cochliomyia*. This failure to observe ovarian tumors can be attributed to the fact that such tumors are relatively rare (in the *Drosophila* experiments only 13 tumorous egg chambers were observed per 10,000 cells examined when females were treated with 4000 r). More important, the two species differ in the pattern of egg production. Ovarian tumors are of clonal origin and arise from oogonial cells that mutate before the formation of a 16-cell cyst. Since the egg chambers examined in the present cytological studies were derived from cells that had probably already undergone the required number of somatic divisions to form a cyst, it was not expected that this type of tumor would be found.

The irradiation of developmental stages in *Drosophila* has resulted in a reduced number of ovarioles, but in *Habrobracon juglandis* (Ashmead) (= *Bracon hebetor* Say), the normal number of ovarioles always forms after treatment of various growth stages (larvae and pupae) with a number of different radiation doses (Erdman, 1961). In this respect, *Cochliomyia* resembles *Habrobracon:* The present studies clearly indicate that the reduction of growth in the ovary is not due to a reduction in the number of ovarioles comprising the ovary, but rather to morphological changes in the ovarioles, which persist in normal numbers.

Failure of mature ova to form is most often associated with a failure of the 15 nurse cells to function normally. However, clear instances in which egg chambers had formed with less than 15 nurse cells were found after irradiation of both 5-day-old pupae and adults 0–4 hours old; in all such instances growth of the egg chamber was seriously hampered. In contrast, King *et al.* (1961) have shown that in *Drosophila,* vitellogenesis in oocytes can proceed with as few as 10 or as many as 30 nurse cell nuclei. It is presumed, however, that for a reduced number of nurse cells to support vitellogenesis, the nurse cells must contain at least the normal polyploid number of chromosomes.

SUMMARY

1. In normal *Cochliomyia hominivorax* females, gross ovarian growth, correlated with the age of the adult from emergence to sexual maturity, was measured. Studies showed that the size of the ovary doubles between the first and second day of adult life, increases more than 5-fold between the second and third day, and exhibits a total increase of approximately 60-fold from emergence to sexual maturity.

2. A cytological study of the ovarioles was conducted to determine the sequence of events in normal oogenesis. The cytology of the reproductive system, from 5-day-old pupae to sexually mature females, is described.

3. The effects of gamma radiation on gross ovarian growth indicated that newly emerged females are more radiosensitive than 5-day-old pupae, and that irradiation of 2-day-old females has little effect on subsequent ovarian growth.

4. The cytopathology of the irradiated ovary was studied after similar doses of radiation were delivered to various developmental stages. The general sequence of events after treatment was as follows: After low doses, growth is slower than normal but not completely arrested. If treatment is given to 5-day-old pupae, grossly malformed oocytes are not often encountered, but second egg chambers frequently do not form. When females 0–4 hours old are irradiated, growth in the first egg chamber is delayed considerably, and is often followed by complete degeneration of the first egg follicle or the formation of grossly malformed oocytes. In these studies, the most radiosensitive stage encountered was that period during which the egg chambers contain nurse cells undergoing endomitotic replications of chromosomal material.

LITERATURE CITED

Baumhover, A. H., A. J. Graham, B. A. Bitter, D. E. Hopkins, W. D. New, F. H. Dudley and R. C. Bushland, 1955. Screw-worm control through release of sterilized flies. *J. Econ. Ent.,* **48**: 462–466.

Bier, K., 1960. Der Karyotyp von *Calliphora erythrocephala* Meigen unter besonderer Berücksichtigung der Nahrzellkernchromosomen im gebundelten und gepaarten Zustand. *Chromosoma,* **11**: 335–364.

Bushland, R. C., 1960. Screw-worm research and eradication. *Advances in Vet. Sci.,* **6**: 1–18.

Bushland, R. C., and D. E. Hopkins, 1953. Sterilization of screw-worm flies with X-rays and gamma rays. *J. Econ. Ent.,* **46**: 648–656.

Erdman, H. E., 1961. Analyses of the differential radiosensitivity of developing reproductive tissues in *Habrobracon juglandis* (Ashmead) to ionizing radiations. *Int. J. Rad. Biol.,* **3**: 183–204.

Fawcett, D. W., S. Ito and D. B. Slautterback, 1959. The occurrence of intercellular bridges in groups of cells exhibiting synchronous differentiation. *J. Biophys & Biochem. Cytol.,* **5**: 453–460.

Grosch, D. S., and R. L. Sullivan, 1954. The quantitative aspects of permanent and temporary sterility induced in female *Habrobracon* by x-rays and beta radiation. *Rad. Research,* **1**: 294–320.

Hirschler, J., 1955. On the cooperation of fusomes in the development of egg-nurse cell complexes in the animal ovary. *La Cellule,* **57**: 67–87.

Jacob, J., and J. L. Sirlin, 1959. Cell function in the ovary of *Drosophila melanogaster*. I. DNA classes in the nurse cell as determined by autoradiography. *Chromosoma,* **10**: 210–228.

Jefferson, M. E., 1960. Irradiated males eliminate screw-worm flies. *Nucleonics,* **18**: 74–76.

King, R. C., 1957. Oogenesis in adult *Drosophila melanogaster*. III. Radiation-induced ovarian tumors. *Growth,* **21**: 129–135.

King, R. C., 1960. Oogenesis in adult *Drosophila melanogaster.* IX. Studies on the cytochemistry and ultrastructure of developing oocytes. *Growth,* **24**: 265–323.

King, R. C., and J. H. Sang, 1959. Oogenesis in adult *Drosophila melanogaster.* VIII. The role of folic acid in oogenesis. *Growth,* **23**: 37–53.

King, R. C., E. A. Koch and G. A. Cassens, 1961. The effect of temperature upon ovarian tumors of the Fes mutant of *Drosophila melanogaster. Growth,* **25**: 45–65.

LaChance, L. E., and A. P. Leverich, 1962. Radiosensitivity of developing reproductive cells in female *Cochliomyia hominivorax. Genetics,* **47**: 721–735.

Lorz, A. P., 1947. Supernumerary chromonemal reproductions: Polytene chromosomes, endomitosis, multiple chromosome complexes, polysomaty. *Botan. Reviews,* **13**: 597–624.

Painter, T. S., 1959. Some values of endomitosis. *In:* Biological Contributions. M. R. Wheeler (ed.), Univ. of Texas Publ. #5914, University Press, Austin, pp. 235–240.

Painter, T. S., and E. C. Reindorp, 1939. Endomitosis in the nurse cells of the ovary of *Drosophila melanogaster. Chromosoma,* **1**: 276–283.

Stalker, H. D., 1954. Banded polytene chromosomes in the ovarian nurse cells of adult Diptera. *J. Heredity,* **45**: 259–264.

PHOTOPERIODIC TERMINATION OF DIAPAUSE IN AN INSECT[1]

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Since the work of Kogure (1933) on the effect of photoperiod on diapause in the commercial silkworm, diapause in many insect species has been found to be photoperiodically induced (see reviews by Lees, 1955; de Wilde, 1962). The European corn borer, *Ostrinia nubilalis,* displays a photoperiodically induced facultative diapause in the final (fifth) larval instar (Mutchmor and Beckel, 1958, 1959; Beck and Hanec, 1960).

Diapause is defined as a state of arrested development in which the arrest is enforced by a physiological mechanism (Beck and Hanec, 1960). Diapause is, therefore, distinguishable from quiescence or dormancy that is enforced by unfavorable environmental conditions. Under natural conditions, diapause is eventually terminated and morphogenesis resumed. The physiological processes involved in the termination of diapause constitute developmental changes on a biochemical level, and have been termed "diapause development" by Andrewartha (1952). Andrewartha defined diapause development as (1952, p. 53) "the physiological development, or physiogenesis, which goes on during the diapause stage in preparation for the active resumption of morphogenesis." It is, therefore, the process of reversing (or replacing) the physiological mechanism enforcing the diapause state.

Experimental work on diapause development has dealt mainly with the low-temperature treatments necessary to terminate diapause; the reviews of Andrewartha (1952) and Lees (1955) discuss many examples of diapause development in eggs, larvae, pupae, and adults. In a few instances, termination of diapause has been found to be photoperiodically induced without a previous exposure of the insects to low temperatures (Baker, 1935; Paris and Jenner, 1959; Shakhbazov, 1961). According to the definitions employed above, photoperiodic termination of diapause must also involve diapause development.

The intensity of diapause, as measured by the length of time required to complete diapause development, varies widely among the species studied; a few days are required for *Loxostege sticticalis* (Pepper, 1937), but several months are needed in the case of *Melanoplus bivittatus* (Church and Salt, 1952). The intensity of diapause may also vary among individuals of the same species, depending on how long they have been exposed to diapause-inducing conditions. De Wilde *et al.* (1959) reported that diapause in the Colorado potato beetle, *Leptinotarsa decemlineata* Say, was photoperiodically reversible shortly after the adults displayed diapause behavior, but not a few days later. Hogan (1962) found that embryonic diapause in the cricket *Acheta commodus* was more intense after 14 days of incubation at 23° C. than after only 7 days at that temperature.

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Babcock (1924) reported that larval diapause in the European corn borer could be terminated only after the larvae had been subjected to about 6 weeks of freezing or near-freezing temperatures. This finding has been generally accepted, and in the absence of any photoperiodic treatment of the diapause larvae, is verifiable. In a recent study, Beck and Apple (1961) reported that diapause could be revoked in laboratory-reared borers by subjecting them to long-day photoperiods shortly after they had entered the diapause state. A low-temperature exposure was not required for such diapause termination, and the authors expressed doubt as to whether or not the larvae had been fully in diapause. They referred to such easily broken diapause as an "incipient" rather than "true" diapause.

The study here presented was undertaken in an effort to determine whether or not diapause in the European corn borer can vary in intensity under different conditions, and also to elucidate the relationship between photoperiod and the completion of diapause development.

Material and Methods

The European corn borers used in this study were from a restricted natural population occurring near Madison, Wisconsin. The use of a defined population was necessary because of the demonstration of significant differences in photoperiodic responses among different geographical populations of this species (Beck and Apple, 1961). Overwintering borers were collected from the field in the fall of the year, and were stored at 5° C. As needed, groups of stored borers were incubated at 30° C. for pupation and emergence. The progeny of these insects were used in the experiments described below, except where field borers are indicated. The times of collection and storage conditions for field borers are indicated in the appropriate sections.

The laboratory borers were reared aseptically on purified diets according to the rearing techniques described by Beck and Smissman (1960). All experiments were run at 30° C. with the exception of those treatments involving a temperature cycle or storage at 5° C.

The experiments were carried out in B.O.D. constant temperature incubators that had been modified to incorporate a thermistor temperature control system (Thermistemp Temperature Control Model 71, Yellow Springs Instrument Company, Yellow Springs, Ohio). Control of photoperiod was effected through the use of 7-day cycle programmers wired to two 14-watt fluorescent lights installed in the incubator. In experiments involving temperature changes, two methods were used. Symmetrical temperature cycles were obtained by using a clock motor to drive the thermistor temperature control unit through a prescribed cycle. The temperature reached a maximum of 31° C. and 12 hours later reached a minimum of 21° C. A temperature cycle with abrupt changes was obtained by having the thermistor temperature controller switched from 32.5° C. to 12° C. by a 24-hour programmer. This apparatus was set to give a temperature cycle with 16 hours at 32.5° C. and 8 hours at 12° C. The change from the maximum temperature to the minimum temperature took 1½ hours, while the change from the minimum to the maximum temperature took one hour. The performance of the temperature-controlling apparatus was verified by a recording thermograph.

Throughout this paper the term *short-day* refers to a photoperiod consisting of 13 hours of photophase and 11 hours of scotophase, and the term *long-day* refers to a photoperiod with a 16½-hour photophase and a 7½-hour scotophase. These experimental conditions were employed because previous work had shown that over 90% incidence of diapause was induced in the Madison population of the European corn borer when they were grown under a 12–13-hour photophase in a 24-hour photoperiod. Either more or less light induced less diapause. There was no appreciable diapause in response to a long day, continuous light, or continuous dark (Beck and Hanec, 1960).

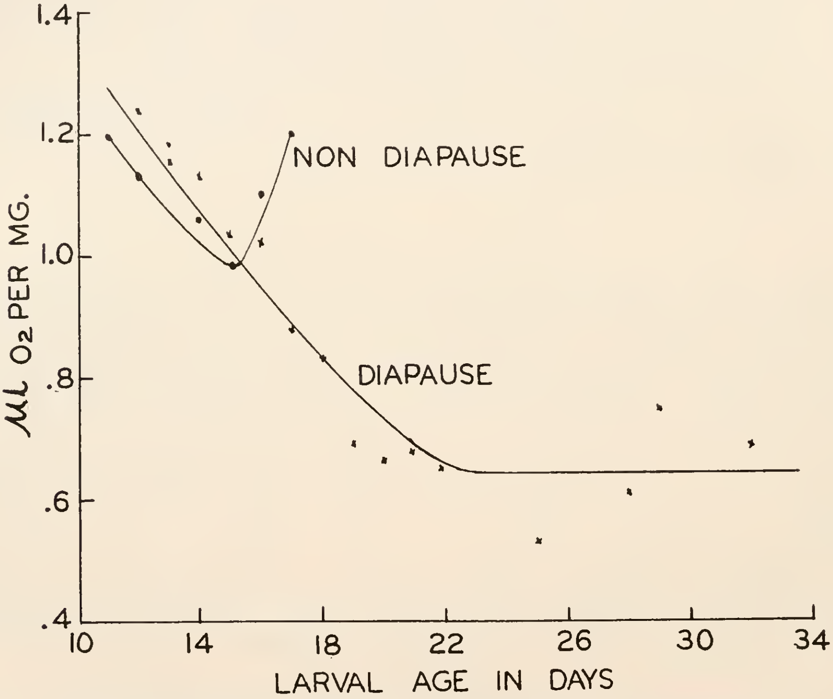

FIGURE 1. Rate of oxygen consumption by diapause and nondiapause larvae of the European corn borer.

Diapause in the individual borer was determined by a negative criterion—failure to pupate. With such a criterion, there had to be an arbitrary point of time selected, after which the borers were considered to be in diapause. This point was reached experimentally when the control borers, reared in the dark, had finished pupating and the pupation curve for the experimental population had leveled off (see Figure 2). Diapause borers were obtained for experimentation by rearing them in a short day for 21 days after eclosion. At this time they were placed in clean vials on wet paper strips. Experiments on breaking diapause were terminated when all the borers in the sample had either pupated or died.

The oxygen consumption of laboratory-reared borers was carried out using standard manometric techniques (Umbreit *et al.,* 1957). Borer larvae were confined in small wire cages in each Warburg flask. This prevented them from crawling into the center well and, because the insect is thigmotactic, also tended to keep them relatively inactive. The oxygen consumption of each larva was measured for one hour in 10-minute increments. The μl. O_2 consumed per hour was divided by the live weight of the insect to give μl. O_2/mg./hr.

The analyses of variance were calculated by the method of Steel and Torrie (1960) for groups with unequal replication. Duncan's New Multiple Range test was used with the approximation of Kramer (cited in Steel and Torrie, 1960) for testing means based on unequal replication.

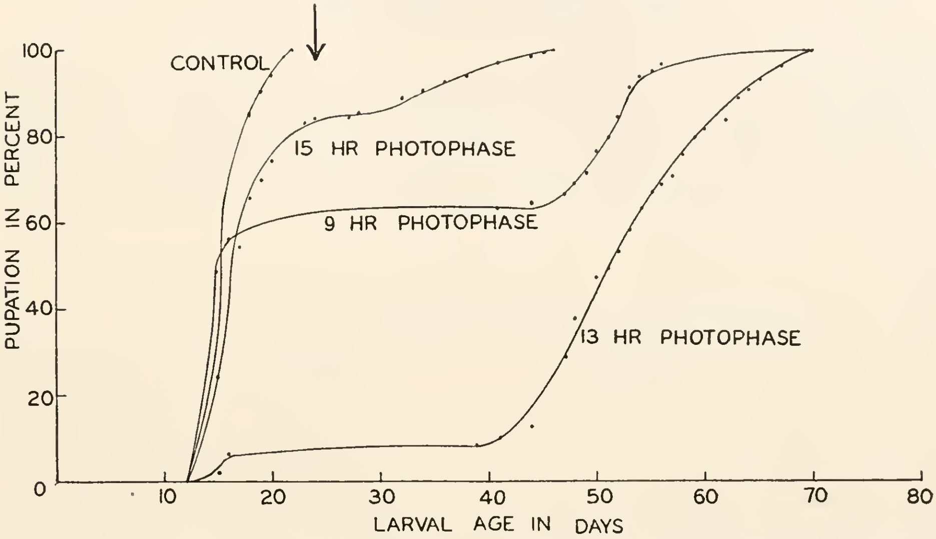

FIGURE 2. Pupation curves for European corn borer populations reared under different photoperiods. The arrow indicates the time at which all diapause larvae were exposed to long-day photoperiods (16.5L/7.5D).

RESULTS AND DISCUSSION

The diapause stage in most insects is characterized by a low level of oxygen consumption (Heller, 1926; Boell, 1935; Schneiderman and Williams, 1953). Beck and Hanec (1960) found that the respiration of borers collected from the field also dropped, but little was known about the length of time necessary to reach this low level of oxygen consumption as the borers entered diapause. The "incipient" diapause reported by Beck and Apple (1961) may have reflected an incomplete suppression of respiration. Once a stable low level of oxygen consumption was reached, the "true" diapause stage would have been attained.

Figure 1 shows that oxygen consumption declined after the moult from the fourth to the fifth instar in both diapause and nondiapause borers. The nondiapause respiration increased at the pupal moult to form the classical U-shaped curve. Respiration in the diapause larvae continued to drop, stabilizing at from one half to one third the prediapause level. If a stable low level of oxygen consumption is indicative of diapause, then the diapause stage had been reached at this time (22–24 days). If diapause occurred in different intensities after this time, measurement of its intensity would require some method other than oxygen consumption. The average time to pupation when placed in a long day was the criterion used to determine the intensity of diapause in subsequent experiments, the supposition being that the more intense the diapause, the greater the delay in pupation.

The intensity of diapause in larvae of the European corn borer was tested in larvae reared under different photoperiods (Fig. 2). Diapause incidence of greater than 90% was observed among larvae reared under a photoperiod consisting of a 13-hour photophase and an 11-hour scotophase. Intermediate incidence of diapause was obtained under a 15-hour photophase and a 9-hour scotophase and also under a 9-hour photophase and 15-hour scotophase. The 15-hour photophase is on the long-day side of the response maximum, and the 9-hour photophase is on the short-day side of the response peak (Beck, 1962).

Table I

The average time to pupation of diapause borers grown under different photophases when transferred to a long day

Photophase (hr./24 hours)	Average time to pupation
9	29.9
13	29.8
15	13.5*

* This mean is significantly different from the other means at the 5% level of probability.

Larvae reared under the 9- and 13-hour photophase treatments reached a maximum incidence of pupation at 15–16 days, and the pupation curve leveled off, while the 15-hour treatment leveled off more slowly (24 days). The dark controls had finished pupating by 22 days. The non-pupators in the three treatments were then considered to be in diapause and were placed in a long-day photoperiod.

When exposed to a long-day photoperiod, the borers reared under the 9- and 13-hour photophase began to pupate in 17–19 days. At from 65 to 70 days of age, all had pupated. The 15-hour photophase treatment, on the other hand, did not result in this pattern. Diapause in these larvae did not appear to be as intense as in the other two groups, and pupation was nearly finished by the time that pupation had started in the other two experimental populations. The average times to pupation, as shown in Table I, were also significantly lower. Different intensities of diapause occurred under different photoperiods, but long-day exposures terminated diapause in borers grown under any of the three photoperiods tested.

There still remained the possibility that longer exposure to a short day would induce a more intense diapause, as shown by de Wilde *et al.* (1959) and Hogan (1962). To test this hypothesis, diapause was induced in a large group of borers with a short-day photoperiod. At 30 days of age, and at subsequent 20-day intervals to 90 days, samples were removed and placed in either continuous dark or

TABLE II

The average time to pupation of diapause larvae sampled at different ages from a population held continuously under a short day

Larval age at transfer (days)	Average time to pupation: Cont. dark (days)	Average time to pupation: Long day (days)
30	78 (2)*	30.7 (23)
50	29 (3)	30.1 (12)
70	17 (3)	21.7 (12)**
90		16.1 (14)**

* Numbers in brackets refer to the number of pupae/sample of 30.
** This mean significantly different from the rest at the 5% level.

a long-day regime, and observed daily for pupation (Table II). Diapause was terminated at any age, and the average time to pupation became shorter with increasing age. The average time to pupation in the 70- and 90-day samples transferred to the long day may have been shortened partly because some of the borers were close to pupation before the long-day treatment was begun.

Diapause in the corn borer does not last indefinitely, even under diapause-inducing conditions. The borers that remained under the diapause-inducing short day started to pupate at about 70 days of age, and by 140 days 36% had pupated and 64% had died. This finding can, perhaps, be explained on the basis that diapause development may proceed at 30° C. Diapause development then proceeds to completion in about one third of the population by 140 days. This would mean that diapause in the borer is much the same as that in *Philosamia cynthia* (Danilyevsky, 1949; cited by Lees, 1955). The high end of the temperature range for diapause development corresponds with a large part of the temperature range for morphogenesis. Although diapause development can occur at 30° C., the high rate of mortality and the length of time for pupation would indicate that this is not the optimum temperature for diapause development. It is probable that mortality occurs because the borers have utilized their fat body reserves before diapause development has been completed. An alternative hypothesis is that a diapause of sufficient intensity was not attained under the experimental conditions.

Experiments were set up to determine if a more intense diapause could be induced by varying the temperature and photoperiod to which the insects were exposed. Diapause was induced in two groups of borers, and they were placed in

TABLE III

The average time to pupation of diapause borers stored in continuous dark at 30° C. when transferred to a long day

Larval age at transfer (days)	Days in dark	Average time to pupation in long days (days)
21	0	29.3 (24)*
35	14	29.0 (22)
49	28	24.2 (25)
63	42	28.0 (18)
77	56	27.1 (16)

* Numbers in brackets refer to the number of pupae/sample of 30.

continuous dark at either 30° C. or 5° C. when they were 22 days old. Periodically samples were removed and placed in a long day, and the average time to pupation determined. Tables III and IV show that neither continuous dark nor cold treatment induced a more intense diapause. Diapause was terminated by a long day, and there was no requirement for chilling. The F value from the analysis of variance for the data in Table III was not significant at the 5% level. Table IV shows that the long-day treatments yielded significantly different results. The borers that were exposed to 5° C. for 42 days took less time to pupate than any of the other three groups. But it can be seen from the continuous-dark column (Table IV) that diapause development was completed in very few of the borers during the exposure to 5° C.

In the field, corn borers are not exposed to a constant temperature as in the incubator but to a fluctuating temperature with a low during the night and a high during the day. Beck (1962) found that 96% diapause was induced when borers were grown under a short-day photoperiod and a thermoperiod with the cold during the dark. If the cold came during the light, only 15% diapause was induced.

Table IV

The average time to pupation of diapause borers stored in continuous dark at 5° C. when transferred to a long day or continuous dark at 30° C.

Larval age at transfer (days)	Days in dark (5° C.)	Average time to pupation in:	
		Cont. dark (days)	Long day (days)
21	0	— (0)*	27.2 (21)
35	14	52.4 (5)	28.1 (18)
49	28	43.0 (3)	30.7 (9)
63	42	33.0 (1)	19.4 (12)**

* Numbers in brackets refer to the number of pupae/sample of 30.

** This mean is significantly different from the others at the 5% level of probability.

Diapause was induced in a short-day and either a constant temperature (26° C.) or a 21–31° C. symmetrical thermoperiod. The average time required for pupation was compared in the two groups by samples taken at two different times (26 and 50 days). The F values from the split plot analysis of variance were not significantly different at the 5% level for the long-day treatments in Table V. Thus, a thermoperiod imposed on a photoperiodic regime did not induce a more intense diapause.

A diapause incidence of about 45% was induced when borers were reared in continuous dark and a thermoperiod consisting of 16 hours at 32.5° C. and 8 hours at 12° C. with abrupt temperature changes. Borers were reared and held under these conditions for 90 days. Periodically samples were removed and placed in either continuous dark or long day, and observed for pupation. Table VI shows the average time to pupation for these groups. The analysis of variance for the long-day treatments gave a non-significant F value at the 5% level, and it is concluded that a thermoperiod alone does not induce a more intense diapause.

TABLE V

The average time to pupation of borers grown under a short day and either (A) 26° C. constant temperature, or (B) 21–31° C. symmetrical thermoperiod when transferred to a long day or continuous dark

Larval age at transfer (days)	Temperature condition	Average time to pupation	
		Cont. dark (days)	Long day (days)
26	A	108 (1)*	29.8 (25)
26	B	68 (1)	28.2 (23)
50	A	41.5 (5)	28.3 (27)
50	B	59.8 (5)	33.1 (18)

* Numbers in brackets refer to the number of pupae/sample of 30.

The preceding data show that an intense diapause, that is, a diapause that required a prolonged period of chilling for termination, could not be induced. Diapause could always be terminated with a long-day photoperiod. There was no requirement for chilling to break diapause.

Experiments on the response of field-collected borers to a long-day photoperiod were begun at the end of August when pupation was completed in the field population. Samples of borers were collected periodically and placed in either a long day or in continuous dark. Table VII shows the average time to pupation for these groups. The results show that diapause could be broken in the field population by a long day, and that diapause termination did not require chilling. It is evident that the diapause induced in laboratory-reared borers was similar in intensity to that of the field-collected borers.

It is evident from the "continuous dark" column (Table VII) that diapause development had been completed in the majority of borers by December 13. The average time to pupation became shorter after this date, because morphogenesis proceeded slowly at the low temperature in the field. After this date, the average time to pupation in the long day did not differ significantly from that in the dark. Thus, once diapause development had been completed, long-day exposure had no further effect.

The experimental results discussed thus far show that diapause development

TABLE VI

The average time to pupation of diapause borers grown in continuous dark and a thermoperiod of 16 hours 32.5° C. and 8 hours 12° C. when placed in continuous dark or long day

Larval age at transfer (days)	Average time to pupation	
	Cont. dark (days)	Long day (days)
45	68.3 (3/15)*	31.0 (13/15)
60	—	31.7 (18/30)
75	—	26.9 (17/30)
90	21.6 (5/15)	23.1 (14/25)

* Numbers in brackets refer to the number of pupae/number in sample.

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can proceed very slowly to completion at 30° C. in continuous dark or under a short day. Long-day treatment greatly accelerates the rate of diapause development. The mortality that occurred when borers were held for a long period of time at 30° C. indicates that borers may need chilling, not to complete diapause development, but rather to decrease the metabolic rate so that there is enough fat body reserve to insure morphogenesis once diapause devlopment has been completed.

Since diapause development can be completed in the corn borer by a long-day photoperiod, a question arises as to the number of days of long-day treatment necessary. To answer this question, diapause was induced in a group of borers by rearing them under a short-day photoperiod for 21 days; they were then placed in a long-day incubator. Periodically samples were removed and placed in continuous dark and the percentage pupation observed at 66 days of age. The experiment was terminated at this age because pupation was finished in the group that remained in the long day, and pupation could occur after 70 days among diapause borers held in the dark.

TABLE VII

The average time to pupation of diapause field-collected borers when placed in either a long day or continuous dark

Date collected	Average time to pupation	
	Long day (days)	Cont. dark (days)
Aug. 22	24.7 (25/27)*	71.1 (9/24)
Sept. 6	24.6 (28/30)	93.5 (4/6)
Sept. 20	25.9 (22/30)	124 (1/6)
Oct. 4	27.7 (26/30)	60.0 (2/6)
Oct. 18	24.9 (18/21)	— —
Nov. 1	27.0 (24/30)	73.6 (7/21)
Nov. 15	27.4 (27/30)	57.7 (4/21)
Nov. 29**	24.0 (3/30)	29.0 (1/30)
Dec. 13	26.8 (25/36)	26.6 (17/36)
Dec. 27	21.2 (20/30)	26.9 (14/30)
Jan. 10	16.7 (21/30)	20.2 (17/30)
Jan. 24	14.9 (21/30)	16.6 (24/30)
Feb. 7	15.1 (21/30)	17.4 (17/30)
Apr. 24	11.8 (22/27)	11.8 (16/27)

* Numbers in brackets refer to the number of pupae/sample size.

** This collection was heavily infected with a fungus and high mortality occurred.

Figure 3 shows that diapause was terminated in 20% of the population by as little as two days of long-day treatment, and with 12–16 days, 100% pupation occurred. It is evident that diapause development induced by the long-day photoperiod took up little of the time (20–30 days) necessary to reach the pupal stage; the remainder of the time was required for morphogenesis.

The terms "diapause" and "diapause development" have been used in this discussion with no attempt to assign these terms to any physiological mechanism, although experimentally these processes have been shown to occur. Wigglesworth (1934) was the first to propose that diapause was linked to the hormones controlling growth and metamorphosis. Subsequently, Williams (1946, 1947, 1948) showed that diapause in the Cecropia silkworm was caused by the failure of the neurosecretory cells of the brain to secrete the activation hormone. The brain regains its ability to secrete the activation hormone when chilled for 6 weeks at 5° C. The activation hormone stimulates the prothoracic glands to secrete ecdyson, and adult differentiation follows. In this case "diapause" refers to the "metabolic block" preventing secretion of the activation hormone; and "diapause development" to the process taking place at 5° C. that returns the brain to secretory activity. This general scheme has been found to fit the larval diapause in the wheat stem sawfly, *Cephus cinctus* (Church, 1955) and the pupal diapause in the Lime hawk moth, *Mimas tiliae* (Highnam, 1958).

It has been found by Cloutier *et al.* (1962) that the brain of the diapause borer is able to promote adult development in diapause larvae. One of the hypotheses presented to explain this was that the brain contained the activation hormone and that its release was blocked in the intact diapause brain. This situation differs from that found in the giant silkworm, *Hyalophora cecropia*; Williams (1948) found that up to 8 diapause brains could not promote adult development in diapause pupae. Thus, it appears that diapause development in the European corn borer facilitates the release of the product, and not its production.

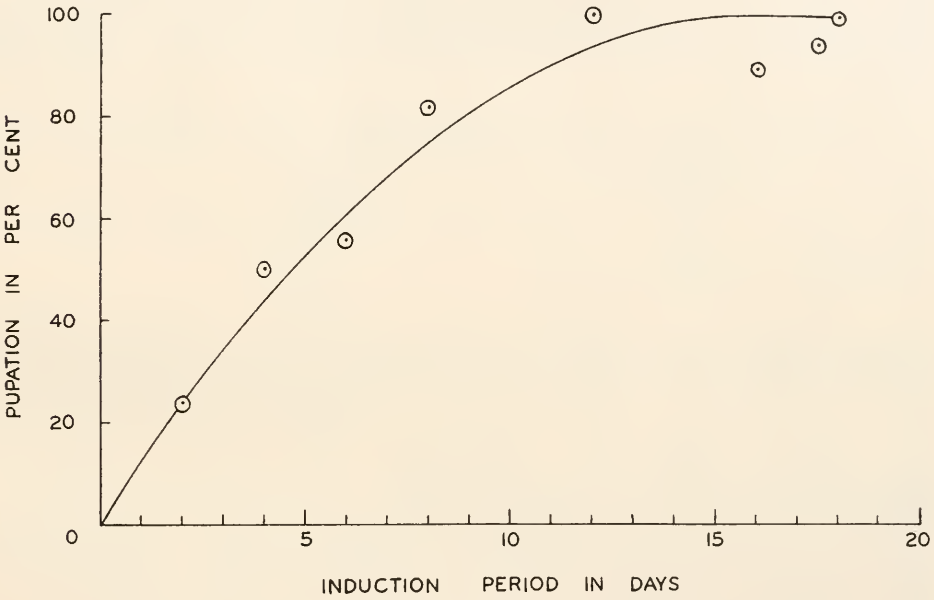

Figure 3. Effect of different long-day exposures on the termination of diapause in the European corn borer.

The sequence of events that appears to take place before pupation can occur is: (1) removal of the block preventing release of neurosecretory products; (2) secretion of the activation hormone in sufficient titer to stimulate the prothoracic glands; and (3) production and secretion of ecdyson in sufficient titer to promote adult differentiation. It is well substantiated that once the prothoracic glands have been stimulated sufficiently by the activation hormone, adult differentiation can proceed

TABLE VIII

The per cent pupation after the re-induction of diapause

Days in long day	Pupation when transferred to Short day %	Pupation when transferred to Cont. dark %
0	0	—
7	0	48
9	33	53
11	67	79
Continuously in long day	100	

without further brain activity (Williams, 1946). This is also true for the corn borer. Of 24 pupae that had their brains removed immediately after pupation, the 22 survivors underwent adult differentiation and emerged as moths. This showed that sufficient activation hormone had been liberated prior to the pupal moult to stimulate adult differentiation, and that the prothoracic glands could be effective in the absence of the brain. If, in terminating diapause with a photoperiodic cycle, secretion of the activation hormone takes appreciable time to reach a level high enough to stimulate the prothoracic glands, there might be some point after diapause development has been completed where the block to secretion might be re-established by a short day. Thus, it could be possible to re-induce diapause by physiologically "removing" the brain.

To test this hypothesis, diapause borers were placed in a long day to induce diapause development. Periodically duplicate samples were removed and placed in either continuous dark or short day. After 45 days from the beginning of the long-day treatment, the percentage pupation was calculated in all groups. Table VIII shows that diapause could be re-induced in all those borers that had only 7 days of long-day treatment and a small percentage could be made to re-enter diapause after 9 and 11 days of long-day treatment. This experiment does not take into account the time necessary to establish the block to neurosecretion, but at least 7 days is not time enough to stimulate the prothoracic glands. But from those samples placed in continuous dark, it can be seen that 7 days of long-day treatment was enough to terminate diapause in 48% of the population. It would thus appear that secretion of the activation hormone is a part of morphogenesis and not diapause development, and hence is not effected by photoperiod.

In the previous experiment the borers were transferred to a short day and remained there until the end of the experiment. As diapause could be reinstated in all after 7 days of long-day treatment, this time was used to determine how many

TABLE IX

The per cent pupation at 45 days when different lengths of time for diapause re-induction were used

Days in long day	Days in short day	Per cent pupation
7	0	46
7	4	40
7	8	20
7	12	6

days of short treatment were necessary to re-induce diapause. This experiment was set up in the same manner as the previous one except that the borers were removed from the short-day incubator at various times and placed in continuous dark. The per cent pupation was calculated in all groups 45 days after the transfer to the long day (66 days of age).

Table IX shows that at least 12 days of short-day treatment are necessary to reduce the per cent pupation from 46% to 6%. It appears, then, that considerable time is required to re-establish the block to neurosecretion. The short length of time necessary for diapause development in a long day compared with the length of time necessary to induce diapause is quite in agreement with what is known about the original induction of diapause (Beck and Hanec, 1960).

Borers collected from the field on October 28 and stored at 5° C. in the dark for 53 days were subjected to different photoperiods. The average times to pupation in Table X were not significantly different at the 5% level. Thus, photoperiod had no effect on these borers. These findings imply that diapause development had been completed, and that morphogenic development had advanced beyond the point where it could be arrested.

Table X

Average time to pupation of field-collected borers under different photoperiods

Photoperiodic condition	Average time to pupation
Continuous dark	18.3
Continuous light	15.4
Long day	15.7
Short day	13.9

SUMMARY

1. The European corn borer, *Ostrinia nubilalis,* has a faculative diapause in the last larval instar. Diapause induced in the laboratory by a short-day photoperiod is identical in its intensity to that occurring in the field.

2. Diapause development occurs at 30° C. under various photoperiodic conditions but is greatly accelerated by a long day.

3. Completion of diapause development does not require a period of chilling.

4. Diapause development is a process that removes a block to secretion of the activation hormone but does not include secretion or any of the morphogenic events that follow.

LITERATURE CITED

Andrewartha, H. G., 1952. Diapause in relation to the ecology of insects. *Biol. Rev.,* **27**: 50–107.

Babcock, K. W., 1924. Environmental studies of the European corn borer (*Pyrausta nubilalis*). *J. Econ. Ent.,* **17**: 120–125.

Baker, F. C., 1935. The effect of photoperiodism on resting tree-hole mosquito larvae. *Canad. Ent.,* 149–153.

Beck, S. D., 1962. Photoperiodic induction of diapause in an insect. *Biol. Bull.,* **122**: 1–22.

Beck, S. D., and J. W. Apple, 1961. Effect of temperature and photoperiod on voltinism of geographical populations of the European corn borer, *Pyrausta nubilalis* (Hbn.). *J. Econ. Ent.,* **54**: 550–558.

BECK, S. D., AND W. HANEC, 1960. Diapause in the European corn borer, *Pyrausta nubilalis* (Hbn.). *J. Ins. Physiol.*, **4**: 304–318.

BECK, S. D., AND E. E. SMISSMAN, 1960. The European corn borer, *Pyrausta nubilalis* (Hbn.), and its principal host plant. VIII Laboratory evaluation of host resistance to larval growth and survival. *Ann. Ent. Soc. Amer.*, **53**: 755–762.

BOELL, E. J., 1935. Respiratory quotients during embryonic development (Orthoptera). *J. Cell. Comp. Physiol.*, **6**: 369–385.

CHURCH, N. S., 1955. Hormones and termination and reinduction of diapause in *Cephus cinctus* Nort. (Hymenoptera: Cephidae). *Canad. J. Zool.*, **33**: 339–369.

CHURCH, N. S., AND R. W. SALT, 1952. Some of the effects of temperature on development and diapause in eggs of *Melanoplus bivittatus* (Say) (Orthoptera: Acrididae). *Canad. J. Zool.*, **30**: 173–184.

CLOUTIER, E. J., S. D. BECK, D. G. R. MCLEOD AND D. L. SILHACEK, 1962. Neural transplants and insect diapause. *Nature*, **135**: 1222–1224.

HELLER, J., 1926. Chemisch Untersuchungen über die Metamorphose der Insekten. III Mitteilung: Über die 'subitane' und 'latente' Entwicklung. *Biochem. Zeitschr.*, **169**: 208–234.

HIGHNAM, K. C., 1958. Activity of the brain/corpora cardiaca system during pupal diapause 'break' in *Mimas tiliae* (Lepidoptera). *Quart. J. Micr. Sci.*, **99**: 73–88.

HOGAN, T. W., 1962. The absorption and subsequent breakdown of urea by diapausing eggs of *Acheta commodus* (Walk.) (Orthoptera: Gryllidae). *Aust. J. Biol. Sci.*, **15**: 362–370.

KOGURE, M., 1933. The influence of light and temperature on certain characters of the silkworm, *Bombyx mori*. *J. Dept. Agric., Kyushu Univ.*, **4**: 1–93.

LEES, A. D., 1955. The Physiology of Diapause in Arthropods. Cambridge University Press, Cambridge.

MUTCHMOR, J. A., AND W. E. BECKEL, 1958. Importance of photoperiod and temperature in inducing diapause in the European corn borer, *Pyrausta nubilalis* (Hbn.). *Nature*, **181**: 204.

MUTCHMOR, J. A., AND W. E. BECKEL, 1959. Some factors affecting diapause in the European corn borer, *Ostrinia nubilalis* (Hubn.). *Canad. J. Zool.*, **37**: 161–168.

PARIS, O. H., AND C. E. JENNER, 1959. Photoperiodic control of diapause in the pitcher-plant midge, *Metriocnemus knabi*. Symposium on Photoperiodism and Related Phenomena in Plants and Animals. (A.A.A.S., Washington, D. C.), pp. 601–624.

PEPPER, H. J., 1937. Breaking dormancy in the sugar beet webworm, *Loxostege sticticalis* L., by means of chemicals. *J. Econ. Ent.*, **30**: 380.

SCHNEIDERMAN, H. A., AND C. M. WILLIAMS, 1953. The physiology of insect diapause. VII. The respiratory metabolism of the Cecropia silkworm during diapause and metamorphosis. *Biol. Bull.*, **105**: 320–344.

SHAKHBAZOV, V. G., 1961. The reaction to the length of daylight and the light receptor of the pupa of the Chinese oak silkworm, *Antheraea pernyi* G. *Doklady Akademii Nauk SSSR, (Biol.)*, **140**: (AIBS translation) 249–252.

STEEL, R. G. D., AND J. H. TORRIE, 1960. Principles and Procedures of Statistics. McGraw-Hill Book Co., Inc., New York.

UMBREIT, W. M., R. H. BURRIS AND J. F. STAUFFER, 1957. Manometric Techniques and Tissue Metabolism. Burgess Publishing Co., Minneapolis, Minnesota.

WIGGLESWORTH, V. B., 1934. The physiology of ecdysis in *Rhodnius prolixus* (Hemiptera). II Factors controlling moulting and 'metamorphosis.' *Quart. J. Micr. Sci.*, **77**: 191–223.

DE WILDE, J., 1962. Photoperiodism in insects and mites. *Ann. Rev. Entomol.*, **7**: 1–26.

DE WILDE, J., C. S. DUINTJER AND L. MOOK, 1959. Physiology of diapause in the adult Colorado beetle (*Leptinotarsa decemlineata* Say). I. The photoperiod as the controlling factor. *J. Ins. Physiol.*, **3**: 75–85.

WILLIAMS, C. M., 1946. Physiology of insect diapause: The role of the brain in the production and termination of pupal dormancy in the giant silkworm, *Platysamia cecropia*. *Biol. Bull.*, **90**: 234–243.

WILLIAMS, C. M., 1947. Physiology of insect diapause. II. Interaction between pupal brain and prothoracic glands. *Biol. Bull.*, **93**: 89–98.

WILLIAMS, C. M., 1948. Extrinsic control of morphogenesis as illustrated in the metamorphosis of insects. *Growth Symposia*, **12**: 61–74.

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ON THE LUNAR ORIENTATION OF SANDHOPPERS (AMPHIPODA TALITRIDAE)

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The sandhopper, *Talitrus saltator,* possesses a mechanism of astronomical orientation which enables it to return by the shortest route to its habitat (the moist area of the beach) when it has been taken away from it. During the day, the animals use the position of the sun to orient (Pardi and Papi, 1952, 1953), while during the night they are able to orient by means of the moon (Papi and Pardi, 1953, 1954, 1959; Papi, 1960). The oriented escape occurs when the animals are placed in unfavorable surrounding conditions. For example, a very low degree of relative humidity or an elevated temperature in the surroundings can act as releasing stimuli. The statistical study of oriented behavior during the return to the sea has been carried out by placing the animals in a concave glass observation chamber on whose dry walls the sandhoppers climb in the direction of the sea.

As early as 1953 we were able to establish that the solar orientation is controlled by an endogenous rhythm and this was confirmed in further research (Papi, 1955; Pardi and Grassi, 1955). More recent research on the lunar orientation (Papi and Pardi, 1959) indicated that a second rhythm, independent of the solar one, may control the angle of orientation with the moon. In fact, even animals kept in constant darkness from sunset onwards or from the new moon preceding the night of the experiment are, for the most part, capable of correct orientation.

As soon as animals are introduced into the observation chamber they orient toward the moon. Only on particularly warm dry nights do the animals change spontaneously from this positive phototactic behavior to a correct orientation toward the sea. It is therefore necessary to heat the observation chamber or to dehydrate the air inside in order to bring about an oriented escape. In the experiments reported in 1959, as well as those in this report, we have always used the method of heating the base of the observation chamber.

Enright has recently (1961) published a paper on the lunar orientation of *Orchestoidea corniculata* in which he arrived at conclusions which differed in part from our own. Enright experimented with animals held in three different sets of circumstances: (A) those kept in constant darkness for ten or more hours before the experiment ("constant darkness"); (B) animals placed in constant darkness at the time of collection and re-exposed to natural light one hour prior to sunset and thus held until the moment of the experiment ("natural light"); (C) animals treated like the former but redarkened two hours after moonrise ("redarkened").

While in the "natural light" and "redarkened" animals Enright found a correct orientation, in the "constant darkness" animals there seemed to be only the tendency to assume a constant angle with the moon, regardless of the lunar stage or position. From this evidence the author drew the conclusion that the existence of a continuously operating endogenous lunar periodicity, which we postulated for *Talitrus,* is not admissible for *Orchestoidea.* Enright suggested that the correct orientation of the "natural light" and the "redarkened" animals could be explained (p. 155) as "a single-cycle night-time orientation rhythm re-initiated by the appropriate stimuli each night."

Concerning the methods used by Enright, we may note that he neither heated the observation chamber nor dehydrated the air inside. In addition, he has observed that in his experimental conditions the repeated use of photo-bulb flashes at short intervals, for making photographic recordings, produced a considerable change in the angle of orientation and in the dispersion of the animals. In the first series of experiments, in each of which three photographs were made, the angle of orientation with the moon tended generally to increase, and there was a similar increase in the dispersion. In two successive experiments, in which ten photographs were made, the author observed instead that (p. 152) "the ultimate angle of orientation with the moon . . . was much smaller than the initial angle."

Since the results of Enright's "constant darkness" animals contrast with those which we obtained from the sandhoppers maintained in darkness from sunset or from the new moon preceding the night of the experiment, and in view of his results on the effects of photo-bulb flashes, which throws doubt upon the validity of the method we employed, we wished to perform a new series of experiments on *Talitrus saltator.*

Materials and Methods

We used *Talitrus saltator* Montagu from the beach at Castiglione della Pescaia (Grosseto) where the theoretical line of escape is 201°. The apparatus was the same as that used for the experiments reported in 1959. The observation chamber was constantly heated, so that the air temperature of the interior was around 22° C.

For the series of experiments, 1, 3, and 4, the animals had been collected the morning before the night of the experiment, between 1000 and 1115. Only for the series of experiments 2 was the collection made in the afternoon (1800–1845), about one hour before sunset. After collection the animals were kept in darkness, in jars containing moist sand, until the moment of their introduction into the observation chamber. The introduction always took place in moonlight and without the aid of artificial light. After five minutes the first photograph was taken, and then eleven others were made at one-minute intervals, except in one case where only ten followed (experiment 4b).

The experiments were performed with a perfectly clear sky on four nights in the summer of 1961. On each night two to four experiments were carried out with as many different groups of animals. For each experiment a variable number of animals (from 16 to over 50) was used, but the number of positions totally recorded (see Table I) is not always a multiple of the number of photographs taken, because of the fact that on some photographs some individuals were not visible (perhaps because the photographs were taken while the animals were jumping). Thus, we obtained single distributions from individual photographs and were able to calculate accumulated distribution.

For each distribution (single or accumulated), we calculated the angular value of the average orientation direction (RD) and the length of the resultant vector, which measures the degree of scatter (OR) (see Pardi and Papi, 1953, p. 463, footnote 1). We have also calculated the average RD and OR for the single distributions, but they are so close to the RD and OR of the accumulated distribution (a maximum difference of ±1° and of ±0.01, respectively), that we have not considered it necessary to report them.

The expected, or theoretical, angle of orientation is the horizontal angle between the moon and the direction to the sea. The observed angle of orientation is the horizontal angle between the average direction of orientation (RD) and the moon. The position of the moon at the mid-point of each experiment was used. In determining these angles we have used for convenience a notation of positive when the moon was to the animal's left and negative when it was to their right.

TABLE I

Orientation of Talitrus saltator with moon

1. Reference number	2. Date	3. Time	4. Lunar stage: days	5. Lunar azimuth	6. No. of recorded positions	7. RD*	8. RD$_1$**–RD	9. Observed angle with moon	10. Theoretical angle with moon	11. Difference, 9–10	12. Vector length (OR)
1a	26 August	$21^h53^m30^s$	16	128°	192	177°	+ 2°	+49°	+73°	−24°	0.78
1b	27 August	$01^h37^m30^s$	16	189°	252	178°	− 1°	−11°	+12°	−23°	0.79
1c	27 August	$04^h10^m30^s$	16	231°	228	205°	− 5°	−26°	−30°	+ 4°	0.80
2a	27 July	$22^h06^m30^s$	16	144°	352	165°	+ 2°	+21°	+57°	−36°	0.74
2b	28 July	$00^h25^m30^s$	16	179°	341	171°	− 3°	− 8°	+22°	−30°	0.66
3a	28 July	$22^h30^m30^s$	17	134°	240	153°	−15°	+19°	+67°	−48°	0.80
3b	29 July	$02^h58^m30^s$	17	204°	191	197°	0°	− 7°	− 3°	− 4°	0.95
4a	3 July	$01^h15^m30^s$	20	126°	300	157°	+ 8°	+31°	+75°	−44°	0.89
4b	3 July	$02^h06^m—^s$	20	137°	585	150°	+ 6°	+13°	+64°	−51°	0.61
4c	3 July	$02^h50^m30^s$	20	148°	192	166°	+ 4°	+18°	+35°	−35°	0.85
4d	3 July	$03^h23^m30^s$	20	157°	299	168°	+12°	+11°	+44°	−33°	0.68

* RD: The resultant direction of orientation calculated by means of the complete distribution.

** RD$_1$: The resultant direction of orientation calculated from the registered distribution of the first photograph.

RESULTS

1. *Effects of the photo-bulb flash*

In each experiment, the variations of the RD of the OR in the single distributions, as compared with the RD and the OR of the accumulated distributions, are represented in Figures 1 and 2. The oscillations of RD are modest, with a difference between maximum and minimum at the most less than 20°. In one case (3a)

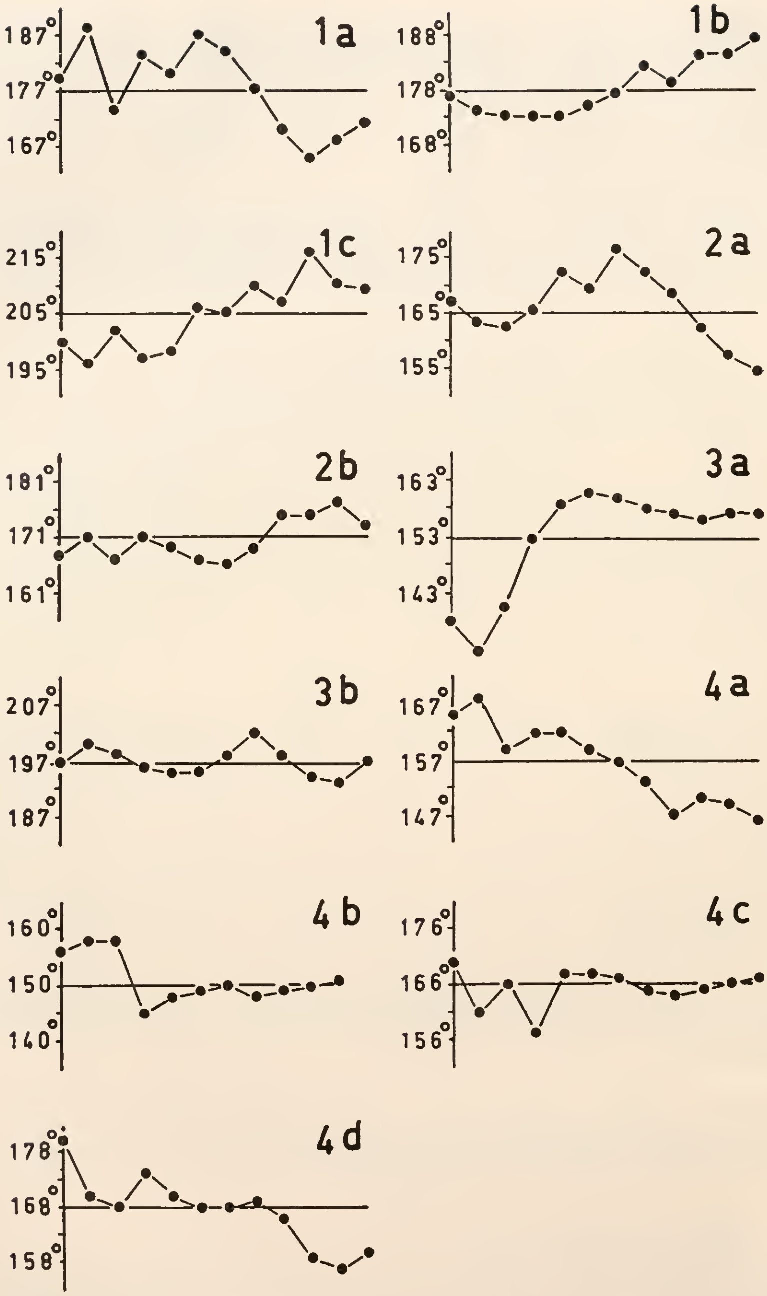

FIGURE 1. The resultant directions (RD) in each experiment. The horizontal line in each graph indicates the value of the resultant direction calculated by means of the accumulated distribution; circles indicate the RD of the single distributions.

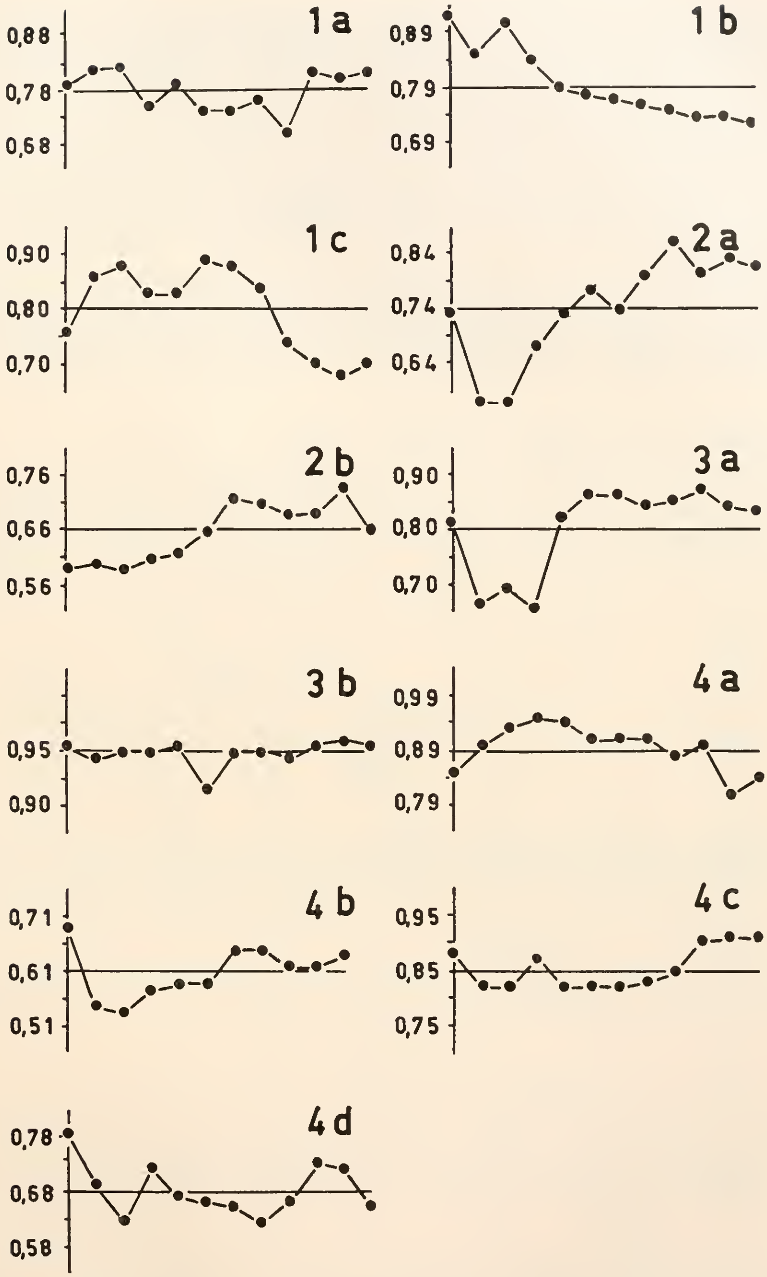

Figure 2. The degree of scatter (OR) in each experiment. The horizontal line in each graph indicates the OR calculated by means of the accumulated distribution; circles indicate the OR of the single distributions.

there is an oscillation of 29°, but it seems clear that the animals, during the first three photographs, still showed the positive phototactic behavior which precedes the correct orientation (the lunar azimuth was 134°). It may be noted that the variations of the RD do not seem to follow a fixed pattern. The differences between the RD of the first single distribution and the RD of the accumulated distribution are also shown in Table I. The algebraic average of these differences is less than 1° ($\overline{X} = +0°54' \mp 7°12'$) and consequently we retained, as already stated, the use of the RD of the accumulated distribution as a valid index of the animals' orientation.

The degree of the scatter (Fig. 2) does not vary considerably, nor in any regular pattern. It should also be noted that the OR is always greater than the value of 0.50 which Enright considered the minimal value having significance when calculated for groups of 20 or 30 animals.

2. *The variations of the angle of orientation*

In general the animals seemed to be well oriented, since only in two experiments out of eleven did the RD of the accumulated distribution differ more than 45° from the theoretically expected direction. Moreover, the degree of scatter, as we have seen, was always so small that the distributions must be considered

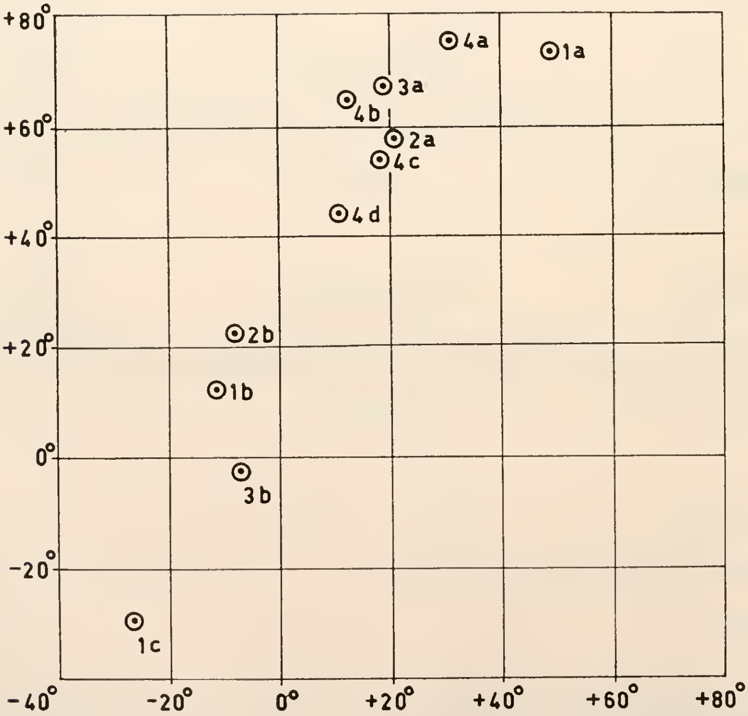

FIGURE 3. Correlation between the theoretical angle of orientation (ordinate) and the observed angle of orientation (abscissa).

significant. In comparison with animals of this same population tested immediately after capture (Papi and Pardi, 1959, p. 587), the results of the current experiments show a stronger tendency to deviate towards south and southeast, a tendency which is generally more marked the nearer the moon is to the east.

A fact of critical importance is whether the angle of orientation with respect to the moon varies in relation to the moon's position or tends instead to oscillate randomly around some other value. Figure 3 represents the correlation between the observed angles and the theoretical angles. The graph indicates a good correlation by inspection. The coefficient of correlation is $r = 0.83$ ($t_r = 4{,}462$; $P < 0.01$). The observed angles of orientation are positive in the first part of the night, tend to become smaller, and then negative, as the moon approaches the west (Table I, col. 9 and Fig. 4). Thus, there is a regular variation in the angles, except for the one night of 3 July. We have not noticed any difference in the orientation or in the dispersion between animals collected in the morning (series of experiments 1, 3, and 4) and in the afternoon (series of experiments 2).

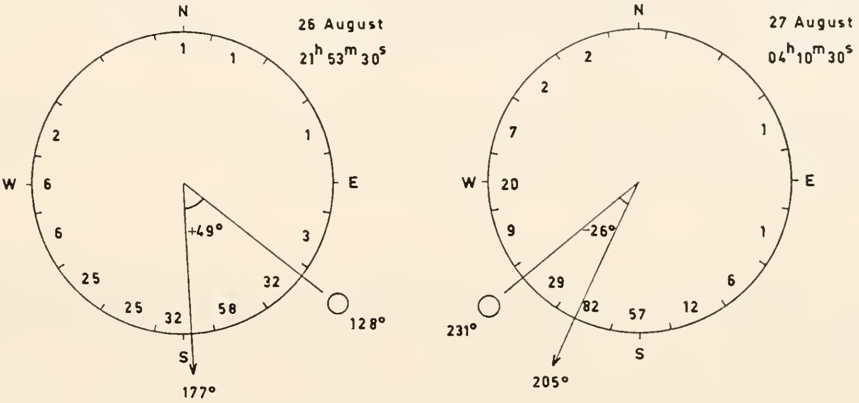

FIGURE 4. An example of the variation of the angle of orientation with moon shown by two lots of animals in the course of the same night. Each number inside the circle shows the number of positions recorded in that sector. The open circles outside represent the moon; the arrows indicate the resultant directions.

DISCUSSION

From the results of the above mentioned experiments, we may observe that: (1) the repeated use of the photo-bulb did not produce any noticeable disturbances; (2) the animals continued regularly to vary the angle of orientation, even when they were not exposed to natural light variations resulting from the sunset and from the moonrise.

The stronger tendency to deviate towards the south and southeast, in comparison to our former experiments in which animals were tested immediately after capture, may be attributed to the interruption in the natural light cycle. This could have induced a small shift in the endogenous mechanism of lunar orientation without, however, having arrested its functioning.

We are able, therefore, to confirm the validity of our previous results on *Talitrus saltator* (1953, 1959) and do not find, for this animal, support for Enright's hypothesis that the sunset and/or the moonrise can start (p. 155) "a single cycle of appropriately time-compensated lunar orientation." The hypothesis of an endogenous lunar periodicity, which operates continuously, still seems the most plausible.

The differences between our results and those of Enright could be due to different mechanisms of orientation in the two species. We think it more likely, however, that they are due to the fact that since Enright did not heat the observation chamber nor dehydrate the air inside, the releasing stimuli on certain nights did not attain the necessary threshold. It should be noted that all the experiments with animals "not kept in constant darkness" (Enright's Table II) were made in a single night (6–7 August) and that on the same night even animals "kept in constant darkness" (Table I, last five experiments) oriented with nearly the same degree of precision. It is therefore probable that new experiments on *Orchestoidea* could explain the difference between Enright's results and ours.

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SUMMARY

1. Under the action of appropriate releasing stimuli the amphipod, *Talitrus saltator,* is capable of orienting by the position of the moon in a relatively constant azimuth. This ability still functions after 10 or more hours of captivity in constant darkness.

2. The direction of escape and the degree of scatter are not noticeably influenced by the repeated photo-bulb flashes.

3. Additional evidence supports the hypothesis that the lunar orientation of *Talitrus* is due to a continuously-operating lunar physiological rhythm. The hypothesis of a single-cycle night-time orientation rhythm, put forward for *Orchestoidea corniculata,* does not seem applicable to *Talitrus saltator.*

LITERATURE CITED

ENRIGHT, J. T., 1961. Lunar orientation of *Orchestoidea corniculata* Stout (Amphipoda). *Biol. Bull.,* **120**: 148–156.

PAPI, F., 1955. Experiments on the sense of time in *Talitrus saltator* (Montagu) (Crustacea-Amphipoda). *Experientia,* **11**: 201.

PAPI, F., 1960. Orientation by night: the moon. *Cold Spring Harbor Symp. Quant. Biol.,* **25**: 475–480.

PAPI, F., AND L. PARDI, 1953. Ricerche sull'orientamento di *Talitrus saltator* (Montagu) (Crustacea-Amphipoda). II. Sui fattori che regolano la variazione dell'angolo di orientamento nel corso del giorno. L'orientamento di notte. L'orientamento diurno di altre popolazioni. *Zeitschr. vergl. Physiol.,* **35**: 490–518.

PAPI, F., AND L. PARDI, 1954. La luna come fattore di orientamento degli animali. *Boll. Ist. Mus. Zool. Univ. Torino,* **4**: 1–4.

PAPI, F., AND L. PARDI, 1959. Nuovi reperti sull'orientamento lunare di *Talitrus saltator* Montagu (Crustacea-Amphipoda). *Zeitschr. vergl. Physiol.,* **41**: 583–596.

PARDI, L., AND M. GRASSI, 1955. Experimental modification of Direction-finding in *Talitrus saltator* (Montagu) and *Talorchestia deshayesei* (Aud.) (Crustacea-Amphipoda). *Experientia,* **11** : 202–210.

PARDI, L., AND F. PAPI, 1952. Die Sonne als Kompass bei *Talitrus saltator* (Montagu) (Amphipoda-Talitridae). *Naturwiss.,* **39** : 262–263.

PARDI, L., AND F. PAPI, 1953. Ricerche sull'orientamento di *Talitrus saltator* (Montagu) (Crustacea-Amphipoda). I. L'orientamento durante il giorno in una popolazione del litorale tirrenico. *Zeitschr. vergl. Physiol.,* **35**: 459–489.

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THE EFFECT OF P-CHLOROMERCURIBENZOATE ON AMOEBOID MOVEMENT, FLAGELLAR MOVEMENT AND GLIDING MOVEMENT

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It has been shown (Abe, 1959, 1963) that the rotational protoplasmic streaming observed in plant cells is dependent upon sulfhydryl (–SH) groups in the protoplasm. In addition, De Robertis and Peluffo (1951) reported an intimate relation between the motility of a flagellated bacterium, *Proteus vulgaris*, and –SH groups. Therefore, it seemed worthwhile to investigate the relationship between –SH groups and other types of protoplasmic motility. Amoeboid movement, ciliary or flagellar movement and gliding movement have been dealt with in this report.

Materials and Methods

Amoeboid movement: A strain of *Amoeba proteus* was chosen as an example of an organism exhibiting amoeboid movement. This strain was found to be abundant in cultures of *Elodea*, and was cultivated by the addition of some uncooked rice grains. The specimens were prepared in the following manner.

The amoebae were sucked up, together with the original culture medium, in a pipette and dropped onto the center of a glass slide. In order to avoid mechanical pressure from the coverslip, a pair of thin glass rods about 10 mm. in length and 0.5 mm. in diameter were laid on the slide. Then, the coverslip was put on the drop carefully. Excessive water was removed with a piece of filter paper.

Flagellar movement: Flagellated cells of coenobia of *Pandorina morum* served as a material exhibiting flagellar movement. The material was collected from a rice paddy in a suburb of Osaka.

As these organisms move freely and actively through an aqueous medium, the preparation used for the amoebae is impracticable for their observation. In this case, only one glass rod was used as a support. A coverslip was brought in position after a drop of suspension of coenobia was carefully placed at the center of the slide. A piece of filter paper was next brought in contact with the edge of the coverslip resting on the glass slide, so that only the suspension medium would be sucked off. In this procedure, coenobia in the suspension were carried along with the stream of water and were caught and held in the wedge-shaped space between

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the coverslip and slide. By this means, we obtained a preparation with a slightly slanted coverslip, under which locomotion was prevented, but with which active movement of the flagella could be observed. Whenever exchange of the medium was required, a solution was made to flow in the same direction in order not to refloat the coverslip and not to release the trapped organisms.

Gliding movement: A species of *Oscillatoria* with a comparatively large diameter (about 16 μ) and a species of pinnated marine diatom, *Nitzschia longissima,* were used as materials. The cells of *Oscillatoria* used in the present study are blue-green and disk-shaped, and the ratio of the diameter to the length is about 7. Each filament tip of this species is straight.

The filaments of *Oscillatoria* are well known to show a bending oscillatory motion, but when a short filament (300 μ– 400 μ in length) is isolated free from others, this motion is transformed into one of gliding along the axis of the filament, so that the speed of motion becomes measurable. The speed and direction of the movement of the filament, however, undergo changes even under constant environmental conditions, and temporary cessation often takes place during the movement. Therefore, in order to compare the behavior of the filament under a particular experimental condition with the control, continuous measurements were made for as long as 2–4 hours.

As the motion of *Nitzschia* is slow and takes a fairly straight path, it is not difficult to determine the changes in velocity of locomotion in this organism with an ocular micrometer and stopwatch.

Reagents used throughout this investigation were commercial p-chloromercuribenzoate (PCMB) and L-cysteine supplied from Wako Pure Chemical Industries, Ltd. Since PCMB is only slightly soluble in acid or neutral solutions, it was at first dissolved in solutions of sodium hydroxide and subsequently neutralized with hydrochloric acid. In the present experiments, the sodium concentration was kept at 10^{-2} M, 10^{-3} M and 10^{-4} M according to the concentrations of PCMB. Consequently, for control solutions, 10^{-2} M, 10^{-3} M and 10^{-4} M NaCl solutions were employed. The sodium content of cysteine solutions was made equal to that of the corresponding control solutions. For the marine diatoms, Van't Hoff artificial sea water was used for a basal solution, and solutions of the above reagents were made to contain a concentration of each ion equivalent to that of the artificial sea water.

All of these experiments were performed at room temperature.

Experimental Results

Part I

Experiments were first carried out with solutions of PCMB at different concentrations. Before each experiment, the medium surrounding the organism was removed with a piece of filter paper, and either saline (in cases of *Amoeba, Pandorina* and *Oscillatoria*) or artificial sea water (in the case of *Nitzschia*) was introduced. After having observed the behavior of the organisms under this condition, the solutions were replaced with PCMB solutions containing a certain amount of NaCl or equivalent amount of salts to the Van't Hoff artificial sea water. Concentrations of the PCMB solutions used were 10^{-3} M, 10^{-4} M and 10^{-5} M. The results obtained are summarized in Table I. Detailed data are given in the following text.

TABLE I

The effects of PCMB on amoeboid movement, flagellar movement and gliding movement at different concentrations

Material	Concentrations of PCMB		
	10^{-3} *M*	10^{-4} *M*	10^{-5} *M*
Amoeba	cytolyzed after 10 sec.	cytolyzed after 8 min.	temporary cessation
Pandorina	stopped within several sec.	stopped after 2 min.	no effect
Oscillatoria	stopped within 3 min.	stopped after 10 min.	no effect
Nitzschia		stopped after 1 min.	stopped in 7 min.

Amoeba

(10^{-3} *M*): Immediately after the application of a control solution—10^{-2} *M* NaCl—movement of *Amoeba* became sluggish and the pseudopodia were drawn in. But in a short time, the pseudopodia were re-formed and within 2–3 minutes the organism regained its normal state completely.

After complete recovery, NaCl solution was replaced with 10^{-3} *M* PCMB containing 10^{-2} *M* sodium. Immediately thereafter the boundary between the cell and the external medium became obscure, and the contents of the organism were extruded in about 10 seconds.

(10^{-4} *M*): When the culture medium was exchanged with 10^{-3} *M* NaCl, movement became at first a little sluggish, but it soon recovered. About 30 seconds after admitting 10^{-4} *M* PCMB, the cell gradually became inactive and all pseudopodia were drawn in. Later the organisms became spherical and after 8 minutes they cytolyzed.

(10^{-5} *M*): With 10^{-5} *M* PCMB the effect was much less pronounced. As a control, a solution—10^{-4} *M* NaCl—was first applied in this case, but there was no observable effect. The saline was then replaced with 10^{-5} *M* PCMB containing 10^{-4} *M* sodium. Within 40 seconds all pseudopodia were retracted and locomotion ceased, but in a short time tiny pseudopodia reappeared. In three minutes the motion recovered completely and the organism survived in this medium without any sign of pathological changes.

Pandorina

(10^{-3} *M*): With 10^{-2} *M* NaCl, the flagellar beating of the coenobium remained normal. When the saline was exchanged with 10^{-3} *M* PCMB containing 10^{-2} *M* sodium, flagellar beating came to a standstill in several seconds. Sometimes flagella, which had been beating, were detached from the cell bodies at their basal ends after several seconds.

(10^{-4} *M*): The effect of PCMB on flagellar beating was still to be seen even at a concentration of 10^{-4} *M*; the beating ceased within two minutes.

(10^{-5} *M*): PCMB solution at a concentration of 10^{-5} *M* had no visible effect on flagellar beating.

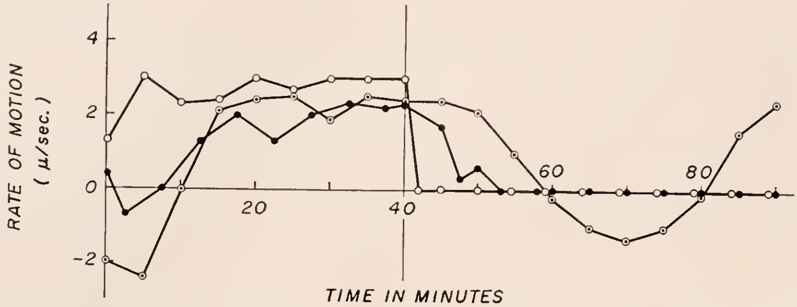

FIGURE 1. The effect of PCMB on the gliding movement of *Oscillatoria* at different concentrations. From 0 to 40 minutes, materials were in NaCl solutions at 10^{-2} *M* (○—○), 10^{-3} *M* (●—●) and 10^{-4} *M* (⊙—⊙); and at 40 minutes, NaCl solutions were replaced with 10^{-3} *M* PCMB + 10^{-2} *M* sodium (○—○), 10^{-4} *M* PCMB + 10^{-3} *M* sodium (●—●) and with 10^{-5} *M* PCMB + 10^{-4} *M* sodium (⊙—⊙).

Oscillatoria

(10^{-3} *M*): As done above, *Oscillatoria* were first placed in NaCl solutions which served as the control. The motion of the filaments, however, was not influenced in NaCl at the concentration of 10^{-2} *M* or less. When 10^{-2} *M* NaCl was replaced with 10^{-3} *M* PCMB containing 10^{-2} *M* sodium, the motion of the filament slowed down and stopped within three minutes and no resumption of motion occurred in the same medium within 20 hours.

(10^{-4} *M*): With 10^{-4} *M* PCMB, the motion decreased, gradually resulting in a complete standstill after 10 minutes. There was no visible change in cell morphology over the observation period of 18 hours.

(10^{-5} *M*): With 10^{-5} *M* PCMB, the gliding motion of the filament was no longer affected at all. These results are illustrated in Figure 1.

Nitzschia

(10^{-4} *M*): The artificial sea water medium was replaced with the artificial sea water containing PCMB. In the plain medium, no change was observable. About

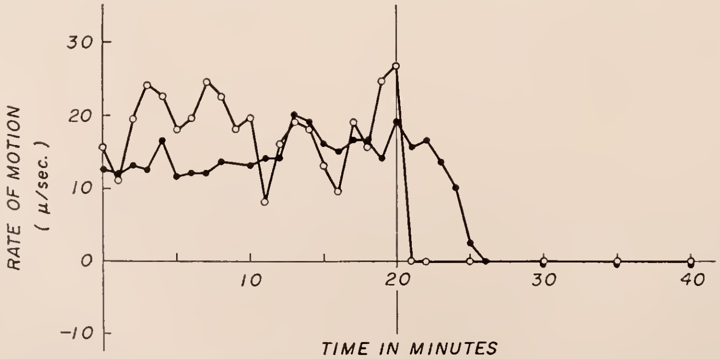

FIGURE 2. The effect of PCMB on the gliding movement of *Nitzschia* at different concentrations. From 0 to 20 minutes, cells were placed in Van't Hoff artificial sea water, and at 20 minutes, medium was replaced with the artificial sea water containing 10^{-4} *M* (○—○) and 10^{-5} *M* (●—●) PCMB.

one minute after the replacement with 10^{-4} M PCMB the gliding motion of the organism fell into complete cessation with no spontaneous recovery (Fig. 2).

(10^{-5} M): Even with 10^{-5} M PCMB, the motion became sluggish and stopped completely in seven minutes (Fig. 2).

Part II

Once the inhibiting concentrations were determined, experiments were carried out to verify the hypothesis that the effect of PCMB was a specific one on sulfhydryl groups. The plan of the experiments with three materials—*Amoeba, Pandorina* and *Oscillatoria*—is illustrated in Table II.

Details of the results of these experiments are given below for each material, the paragraph numbers corresponding to the numbers heading the columns in Table II.

TABLE II

A general outline of the procedure followed and the results obtained in the present experiments. See the text for details

	(1)	(2)	(3)	(4)
TREATMENT and effect	SALINE	SALINE	SALINE	SALINE
	↓	↓	↓	↓
	PCMB	PCMB	PCMB + CYSTEINE	CYSTEINE
	inhibition	inhibition	no inhibition	no visible effect
	↓	↓		
	CYSTEINE	SALINE		
	recovery	no recovery		

Amoeba

(1) About four minutes after treatment with 10^{-4} M PCMB, this solution was replaced with 10^{-2} M cysteine containing 10^{-3} M sodium. Within 60 minutes, no visible change was observed and the cells remained spherical. In such cells, Brownian motion of granules was observed. Then about 70 minutes after replacement, a short pseudopodium was extended, although no conspicuous flow of cytoplasm was observed. After 100 minutes, a pseudopodium with pronounced endoplasmic streaming was formed. By about 160 minutes, complete recovery of amoeboid movement had occurred.

(2) As the control of the former experiment in item (1), PCMB solution was replaced with plain saline instead of a cysteine solution. When 10^{-4} M PCMB solution was replaced, about four minutes after its application with 10^{-3} M NaCl, 2–3 huge vacuoles appeared in the cells. Thirty minutes later, the cells became spherical, and disintegrated in about 40 minutes.

(3) PCMB and cysteine solutions were mixed before they were applied to the organisms. In this mixed solution, when the amount of the latter exceeds that of the former, the –SH combining power of PCMB was expected to vanish. Control solutions—10^{-3} M NaCl—were replaced with a mixed solution containing 5 ×

10^{-5} *M* PCMB, 5 × 10^{-3} *M* cysteine and 10^{-3} *M* sodium. Under these conditions, the organisms behaved quite normally, and there was no observable effect. In support of this statement it is to be added that the amoebae often phagocystosed ciliated cells in this same medium.

For the sake of contrast, a solution containing 5 × 10^{-5} *M* PCMB and 10^{-3} *M* sodium was used. About two minutes after application, movement of the organisms almost stopped except for the Brownian motion of the granules inside. Three minutes later, Brownian motion stopped; six minutes later, the organism became spherical and giant vacuoles appeared; 25 minutes later, the organism began to cytolyze.

Pandorina

(1) Immediately after the complete cessation of the flagellar beating with 10^{-3} *M* PCMB, the inhibitor solution was replaced with 10^{-2} *M* cysteine containing 10^{-2} *M* sodium. Then, recovery of the flagellar beating was observed within several minutes. No abnormal behavior was to be seen later in the organisms which had recovered. Even in the coenobium from which flagella had been partially lost by PCMB, flagella left attached recovered their normal beating.

(2) No recovery of flagellar beating took place when 10^{-3} *M* PCMB was replaced with 10^{-2} *M* NaCl solution.

(3) Materials were treated with a mixed solution of 5 × 10^{-4} *M* PCMB, 5 × 10^{-3} *M* cysteine and 10^{-2} *M* sodium. With this solution, there was no visible effect on flagellar beating.

On the other hand, when treated with a solution containing 5 × 10^{-4} *M* PCMB and 10^{-2} *M* sodium, beating stopped within a period of 20–40 seconds and, sometimes, the flagella separated from cell bodies.

(4) Simple application of 10^{-2} *M* cysteine solution with 10^{-2} *M* sodium normal coenobia exerted no effect.

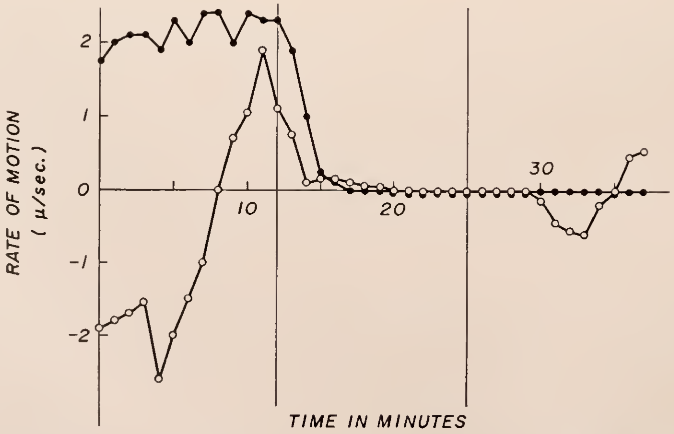

FIGURE 3. Inhibition of the gliding movement of *Oscillatoria* with PCMB and restoration with cysteine. From 0 to 12 minutes, materials were immersed in 10^{-3} *M* NaCl and from 12 to 25 minutes in 10^{-4} *M* PCMB containing 10^{-3} *M* sodium, at 25 minutes, PCMB solution was replaced with 10^{-2} *M* cysteine containing 10^{-3} *M* sodium (○—○) or 10^{-3} *M* NaCl (●—●).

Oscillatoria

(1) About 10 minutes after the complete cessation of gliding motion with 10^{-4} M PCMB, the inhibitor solution was replaced with 10^{-2} M cysteine containing 10^{-3} M sodium. Although in this case a complete recovery was not attained, a partial recovery was observed (Fig. 3).

(2) No sign of recovery was seen in the organism when 10^{-3} M NaCl was substituted for 10^{-4} M PCMB (Fig. 3).

(3) A mixed solution of 5×10^{-5} M PCMB, 5×10^{-3} M cysteine and 10^{-3} M sodium produced no effect, either on motion or on any other aspect of behavior of the organisms.

In contrast to the above, a solution containing 5×10^{-5} M PCMB and 10^{-3} M sodium and no cysteine stopped the gliding motion of the filament within fifteen minutes.

(4) In solutions containing 10^{-2} M cysteine and 10^{-3} M sodium, there was no visible effect on normal organisms.

DISCUSSION

On the basis of the foregoing experiments, it may be concluded that the effect of PCMB on the protoplasmic motility of different types is more or less identical: First, motion becomes sluggish; this is then followed by complete cessation at some particular concentration. Second, motion is restored completely or partially through subsequent application of cysteine in concentrations higher than those of PCMB applied. Third, when the reactivity of PCMB upon $-SH$ groups has been eliminated in advance, through addition of cysteine, PCMB exerts no effect on the cellular motion.

It is well known that PCMB reacts with $-SH$ groups to yield mercaptides. According to Olcott and Fraenkel-Conrat (1947) and Barron (1951), PCMB is the most advantageous of all the known $-SH$ reagents in the following respects: PCMB reacts with $-SH$ groups with high specificity and reacts with no other protein groups; its combination with $-SH$ groups can be thoroughly dissociated with the addition of the reagents having $-SH$ groups in their molecules; these reactions are carried out under physiological conditions. Therefore, it is reasonable to conclude that the cessation and recovery of the movements dealt with in the present paper are closely related to the blocking and liberation of $-SH$ groups in the protoplasm.

In the author's previous work, it was amply verified that the rotational protoplasmic streaming in plant cells has close relation to $-SH$ groups in the protoplasm. In addition, DeRobertis and Peluffo (1951) applied sulfhydryl reagents to the cells of a flagellated bacterium, *Proteus vulgaris,* and reported that the movement of the cells became sluggish and stopped completely under the influence of these reagents, especially PCMB. Recovery of the movement was brought about with subsquent application of cysteine or glutathione, but simple washing with saline or buffer solution did not remove the action of the inhibitors. These results were interpreted by the authors as evidence of the presence of essential $-SH$ groups in the contractile protein of bacterial flagella, or in enzymes especially involved in the mechanism of flagellar motion.

Singer and Barron (1944) showed that PCMB completely inactivated the ATP-ase function of myosin, and subsequent treatment with glutathione restored full activity. Bailey and Perry (1947) have found in addition that the –SH groups of myosin are essential for actomyosin formation. These results point out the significance of –SH groups in muscular contraction. Whether or not the inhibition by PCMB of cellular motions is also due to suppression of ATP-ase activity of a contractile protein is still unknown. But the importance of the –SH groups in the basic mechanism of a variety of protoplasmic motions seems now well established.

The author wishes to express his most cordial thanks to Professor N. Kamiya of Osaka University for his helpful advice and directions throughout this investigation, and to Professor R. D. Allen (Princeton University) for the kind help in preparation of the manuscript.

SUMMARY

1. The effect of p-chloromercuribenzoate (PCMB), a specific sulfhydryl reagent, upon several types of cellular motion—amoeboid movement, flagellar movement and gliding movement—was studied.

2. The effect of PCMB on the motions of different types is more or less identical: First, motion becomes sluggish; this is then followed by complete cessation at some particular concentration. Second, motion is restored completely or partially through subsequent application of cysteine in concentrations higher than those of the PCMB previously applied. Third, when the reactivity of PCMB upon –SH groups has been eliminated with cysteine in advance, PCMB exerts no effect on the cellular motion.

3. From the fact that PCMB is bound to –SH groups with high specificity, it is reasonable to conclude that the cessation and recovery of the cellular motion are closely related to the blocking and liberation of –SH groups.

LITERATURE CITED

ABE, S., 1959. Rotational protoplasmic streaming and SH. *Kagaku* (*Science*), **29**: 361–362 (in Japanese).

ABE, S., 1963. The effect of p-chloromercuribenzoate on rotational protoplasmic streaming in plant cells. In press.

BAILEY, K., AND S. V. PERRY, 1947. Role of sulfhydryl groups in the interaction of myosin and actin. *Biochem. Biophys. Acta*, **1**: 506–516.

BARRON, E. S. G., 1951. Thiol groups of biological importance. *Adv. Enzymol.*, **11**: 201–266.

DE ROBERTIS, E., AND C. A. PELUFFO, 1951. Chemical stimulation and inhibition of bacterial motility studied with a new method. *Proc. Soc. Exp. Biol. Med.*, **78**: 584–589.

OLCUTT, H. S. AND H. FRAENKEL-CONRAT, 1947. Specific group reagents for proteins. *Chem. Rev.*, **41**: 151–197.

SINGER, T. P., AND E. S. G. BARRON, 1944. Effect of sulfhydryl reagents on adenosinetriphosphatase activity of myosin. *Proc. Soc. Exp. Biol. Med.*, **56**: 120–124.

FERTILIZATION IN PECTINARIA (=CISTENIDES) GOULDII

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Pectinaria gouldii was selected for study because the animal was readily available and its egg is more suitable for phase-contrast microscopy that those of many other marine invertebrates, owing to the lack of interfering cytoplasmic structures such as refractile droplets or coarse granulations. The observations are published because, with the exception of Tweedell's (1962) report on cytoplasmic inclusions, only brief notes on *Pectinaria* eggs have appeared in the literature (Wilson, 1936; Costello *et al.*, 1957; Tweedell, 1959, 1960, 1961), and because fertilization in these eggs has several features of interest.

METHODS

The animals were collected in the Woods Hole area during June and July, 1961, and maintained in the laboratory in fingerbowls containing sand and supplied with running sea water. Gametes were obtained by removing an individual to a separate dish and carefully breaking down the conical tube in which the animal lives, starting from the thin end. As a rule, shedding occurred during this process and it was mostly these gametes that were used in the studies to be described. More rigorous methods were often needed to provoke shedding, such as prodding or pinching the animal with forceps, but gametes thus obtained were in the main found to be unsatisfactory, most of the eggs being incompletely grown and the spermatozoa unlikely to leave their packets (Fig. 1) and develop free motility.

Eggs were examined by phase-contrast microscopy, both in the fresh state and after fixation and staining as whole-mounts. A solution of 10% glacial acetic acid in absolute alcohol was used for fixation; staining was effected with acetocarmine (Schneider's). Some eggs in the fresh state were examined by fluorescence microscopy; they were treated with acridine orange solution (0.0005% in sea water) and ultraviolet radiation. Eggs after semination were also fixed in 10% formalin solution, embedded in paraffin, sectioned at 6 μ and stained with haematoxylin and eosin.

OBSERVATIONS

Primary oocyte

The *Pectinaria* egg took the form of a concavo-convex disc 50–60 μ in diameter and 25–30 μ in thickness. It was bounded by a closely applied transparent vitelline membrane about 2 μ thick. The cytoplasm contained numerous finely granular elements and was devoid of large lipoidal droplets.

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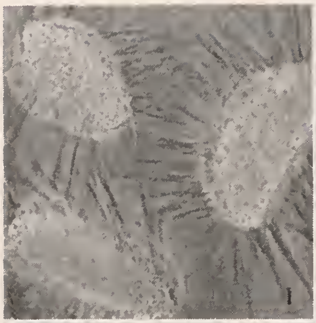

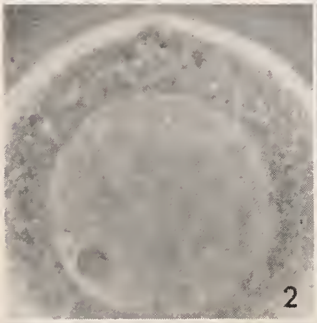

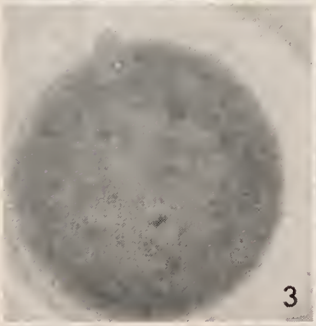

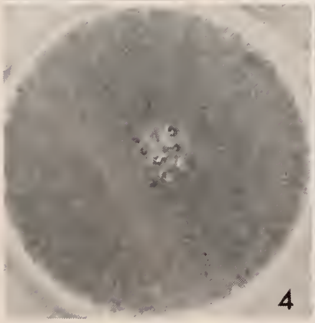

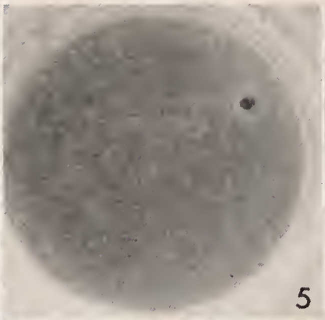

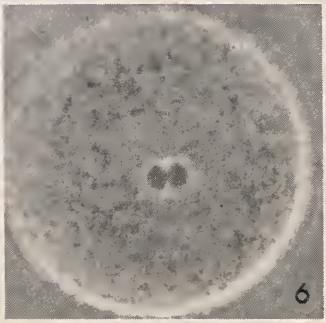

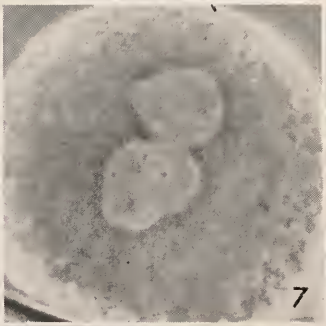

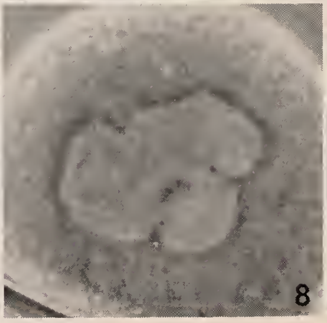

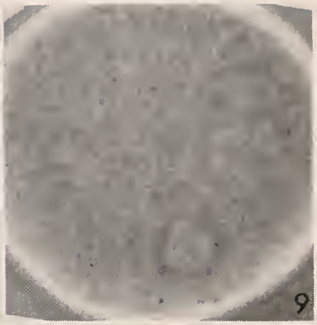

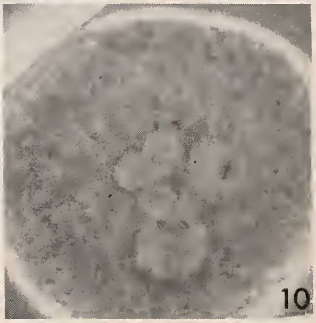

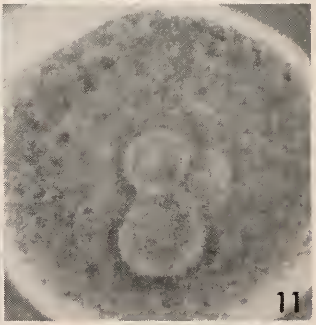

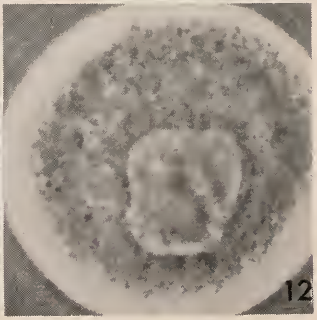

All photographs were taken by phase-contrast microscopy and are reproduced at × 1300.

FIGURE 1. Sperm packets. (Fresh material.)

FIGURE 2. Primary oocyte with intact germinal vesicle. A spermatozoon had recently entered the egg (at top). (Fresh material.)

The germinal vesicle (Fig. 2) was large relative to the size of the egg, its diameter being about half that of the vitellus. A single nucleolus was usually present, in addition to a number of scattered chromosomes. In the living egg, the chromosomes were only vaguely distinguished but they became more evident when they condensed just before breakdown of the germinal vesicle. In the condensed phase of the fixed egg, they could clearly be seen to have the forms characteristic of bivalents. The attempt was made to count the chromosomes in 26 such eggs; estimates ranged from 17 to 21, with a mean of 19.2, uncertainty being due particularly to the presence of several very small chromosomes.

Primary oocytes treated with acridine orange solution and ultraviolet radiation exhibited bright green fluorescence in a zone around the nucleolus and in numerous cytoplasmic granules, and bright red fluorescence in about an equal number of cytoplasmic granules. The nucleolus itself, the nucleoplasm and the hyaline cytoplasm did not have noticeable fluorescence.

Oocytes examined histologically displayed a moderate cytoplasmic basophilia, especially in the granular elements, and a strong basophilia in chromosomes and in the band of material surrounding the nucleolus.

Maturation

The egg was always shed as a primary oocyte; maturation changes began almost immediately, the proportion of eggs showing the changes varying between 10% and 90% in different individuals. The chromosomes condensed, though remaining scattered, the nuclear membrane became irregular in outline and disappeared, and the chromosomes aggregated in the center of the egg as a loose group which became the metaphase plate of the first polar spindle (Fig. 4). Further progress was seen only after sperm penetration; when this occurred, meiosis was resumed and the first and second polar bodies were successively separated. The polar bodies were always extruded into the middle of the concave side of the egg (Fig. 6). The first polar body had a tendency to divide partially or completely, and thus three polar bodies were occasionally seen. Eggs could be left in sea water for at least three hours after shedding without losing their capacity to proceed, on semination, with apparently normal fertilization.

FIGURE 3. A similar egg to that in Figure 2, showing germinal vesicle (with two or three bivalents in approximate focus), a sperm head in the cytoplasm and a residual fertilization cone. (Acetic alcohol and acetocarmine.)

FIGURE 4. Primary oocyte showing polar view of the first meiotic metaphase plate. (Acetic alcohol and acetocarmine.)

FIGURE 5. A similar egg to that in Figure 4, showing a recently penetrated sperm head which had lost its refractility and become stained. (Acetic alcohol and acetocarmine.)

FIGURE 6. Secondary oocyte; surface view showing polar bodies. (Fresh material.)

FIGURE 7. Ootid with male and female pronuclei approaching full development. (Fresh material.)

FIGURE 8. Ootid with two male pronuclei—which appear fused, but were in fact separated by their nuclear membranes—and smaller female pronucleus. (Fresh material.)

FIGURES 9–12. Successive stages of pronucleus development in an egg in which polar body emission failed and two female pronuclei were formed. Seemingly intact nuclear membranes still separated the pronuclei in Figure 12 which give the appearance of having fused. (Fresh material.)

Sperm penetration

The frequency with which sperm packets broke up to liberate free and actively motile spermatozoa differed much between different animals. With good material, complete break-up of the packets occurred within 5 or 10 minutes.

Spermatozoa were occasionally seen to become attached to the vitelline membrane and then pass gradually through this structure; actual entry into the vitellus was not witnessed, the spermatozoa under observation all being stopped apparently at its surface. Many instances were encountered in which a spermatozoon had just entered the egg cytoplasm, and generally the sperm head lay within a small fertilization cone. There was no evidence of elevation of a fertilization membrane. Penetration could apparently occur at any point on the egg surface except in the concave region. A little further progress could be watched until the sperm head became indistinguishable among the cytoplasmic granules. Subsequent stages were studied in fixed and stained eggs, and it was evident that the sperm head enlarged and lost its refractility as it advanced into the egg (Fig. 5). The course of events of entry into eggs before the breakdown of the germinal vesicle was broadly the same as that into eggs of later stages, except that in the former case rather larger fertilization cones were often formed and the sperm head remained refractile and did not enlarge (Figs. 2 and 3). Fertilization proceeded only in eggs penetrated after the germinal vesicle had started to break down. Allowing eggs to stand in sea water for three hours after shedding and before semination did not affect the frequency of sperm penetration.

Pronucleus development

During its early stages of development, the female pronucleus could clearly be seen to be made up of separate portions, karyomeres (Fig. 10), which later came together and fused to form a single nucleus. Evidence of septa could also be seen in the early male pronucleus (Fig. 9). Residual walls or strands tended to persist in both pronuclei at later stages, and seemed to be responsible for irregularities in the outlines of the pronuclei which often looked roughly polygonal (Figs. 7, 8 and 12). At full development, male and female pronuclei came into close apposition and sometimes gave the impression of having undergone fusion (Fig. 12); in no instance, however, could it be said with certainty that actual fusion had occurred, for there always seemed to be a thin membranous structure separating the two nuclei. The chromosome groups deriving from the male and female pronuclei in the prophase of the first cleavage division were regularly found to be quite separate.

Observations indicated that the male pronucleus was formed almost always before the female; it was possible for successful fertilization to be initiated by sperm penetration occurring as soon as the germinal vesicle had broken down, and thus the male pronucleus was formed during the early maturation changes in many eggs. Male pronucleus development then seemed to be suspended until female pronucleus development was under way, for intermediate and later stages of growth were regularly found to be synchronous in the two pronuclei.

In several trinuclear eggs that had two polar bodies (presumptive polyspermic eggs), two pronuclei were seen to be equal in size and both were larger than the third (Fig. 8); in a trinuclear egg that had no polar body (in which it was surmised that two female pronuclei had developed from the egg chromosomes after anaphase separation), two pronuclei were smaller than the third (Figs. 9–12). The inference is that in this species the male pronucleus is larger than the female. The pronuclei contained several small nucleoli (Fig. 7).

Anomalies

The chief anomalies noted were: (1) polyspermy, (2) refertilization, (3) possible early gynogenesis, and (4) failure of polar-body formation. There were no instances of androgenesis or of spontaneous development beyond the first polar metaphase (rudimentary parthenogenesis).

Polyspermy. The occurrence of polyspermy was inferred, in early fertilization, from the presence of two or more sperm heads in the vitellus, or, at later stages, from the presence of three or more pronuclei in eggs having two (or three) polar bodies (Fig. 8). A total of 163 polyspermic eggs was recorded; one was tetraspermic, fourteen trispermic and the rest dispermic. The tetraspermic egg and all but one of the trispermic eggs had intact germinal vesicles. Some eggs observed in stages of the first cleavage division had multipolar spindles; in six, the spindle was tripolar, and in two tetrapolar. These eggs all had two polar bodies and were also assumed to have undergone polyspermic fertilization.

Refertilization. The term is used to denote the entry of a second (or third) spermatozoon into the vitellus much later than the first (or first two), so that the male elements are clearly in different stages of development. The distinction is drawn with polyspermic fertilization in which the male elements are in the same stage of development and apparently advance synchronously. Altogether, ten examples of refertilization were seen, in each of which there was a sperm head that had not undergone noticeable change. Four of these eggs had in addition late-stage male and female pronuclei, two showed an early male pronucleus with the egg chromosomes in the second polar prophase, two had early male pronuclei and egg chromosomes in second polar metaphase, one egg was in the metaphase of the first cleavage division and one egg was two-cell.

Gynogenesis. Possible examples of early gynogenesis were presented by three eggs; each had a well formed female pronucleus and two polar bodies, but the penetrating sperm heads had not appreciably changed.

Failure of polar-body formation. Two eggs were seen in the course of fertilization, both of which lacked polar bodies; they were kept under continuous observation while the pronuclei formed. In one, there were two presumptive female pronuclei and a larger male pronucleus (Figs. 9–12). In the other the sperm head changed into an early male pronucleus, and the egg chromosomes, having failed to undergo anaphase separation, became incorporated into a single female pronucleus, which was about the same size as the male pronucleus.

Time relations and incidence of features of fertilization

The time relations of fertilization were studied in four experiments, the results of which were reasonably consistent; details of the most elaborate experiment are set out in Table I. From these sets of data, the times for fertilization stages were estimated to be as follows: Breakdown of the germinal vesicle became evident a few minutes after shedding and most eggs showed these changes by 10 minutes. The first polar division had advanced to metaphase in a few eggs by 20 minutes and most eggs had reached (or, if penetrated, had passed) this stage within the first hour. Sperm penetration began immediately after semination and virtually all the eggs that were destined to be penetrated contained spermatozoa by 10 minutes after semination. Most of the eggs that were penetrated and lacked the germinal vesicle exhibited first polar bodies and early male pronuclei between 20 and 25 minutes after semination. Second polar bodies and female pronuclei made their appearance within the next 10 minutes. The whole pronuclear phase of fertilization lasted about half an hour, condensation of chromosomes in the prophase of the first cleavage division being evident in most eggs between 50 and 60 minutes after semination. Mitosis seemed to pause in metaphase, and cytoplasmic cleavage was completed in most eggs 20 to 30 minutes later, namely about 80 minutes after semination.

The following inferences are drawn from the observations summarized in Table I. Some eggs fail to undergo any maturation changes, even after standing in sea water for an hour or more: the data show that the proportion of oocytes with intact germinal vesicles fell to 51% (23% + 20% + 8%) at 45 minutes after shedding and suffered only a small reduction thereafter. At each interval from 45 minutes onwards, it was found that roughly half the unchanged eggs had been penetrated by spermatozoa; evidently, sperm penetration neither provoked nor inhibited maturation. The frequency of sperm penetration into primary oocytes, whether before or during maturation, did not change significantly from that observed at 10 minutes after semination—this was despite the presence, for well over an hour, of many active spermatozoa in the medium. The proportion of eggs penetrated was somewhat higher in those with maturation changes (67%) than in those without (54%). On the other hand, the incidence of polyspermy was much higher in eggs with intact germinal vesicle (24% of penetrated eggs) than in eggs at later stages (4% of penetrated eggs).

DISCUSSION

The limits of the breeding season of *Pectinaria* at Woods Hole have not yet been determined, but Costello *et al.* (1957) noted that ripe animals could be secured at least during August. The present investigation was carried out on animals collected in June and July, which may have been before the peak of the season. This would explain why many animals could not be induced to shed, why spermatozoa obtained from others often failed to become free-swimming, and why occasionally as few as 10% of oocytes underwent maturation. The relatively low rate of sperm penetration that occurred (around 60%) despite the presence of many surplus free-swimming spermatozoa may have been owing also to unripeness of animals providing the gametes.

According to Costello *et al.* (1957), polar bodies are separated about 29 minutes after semination, and the first cleavage occurs at about 54 minutes. The corresponding times recorded in the present series were 30–35 minutes and about

TABLE I

Stages of maturation and fertilization in eggs examined at various times after shedding and semination (at 18° C.)

Time after shedding (min.)		15	30	45	60	90	120	150	Eggs		
Time after semination (min.)		—	—	10	25	55	85	115	Total	Penetrated	Polyspermic
Primary oocyte	with germinal vesicle	175 (87)	140 (68)	54 (23)	53 (17)	59 (18)	63 (16)	83 (25)	992	365 (54*)	89 (24*)
	+ 1 sperm			47 (20)	44 (14)	65 (20)	57 (15)	63 (19)			
	+ > 1 sperm			20 (8)	14 (3)	18 (5)	25 (7)	12 (4)			
	late prophase	27 (13)	62 (30)	29 (12)	2 (0.5)	1 (0.3)	3 (1)	1 (0.3)	1015	617 (67*)	16 (4*)
	+ 1 sperm			17 (7)	5 (1.5)	5 (1.5)	1 (0.3)	0			
	+ > 1 sperm			2 (1)	0	1 (0.3)	0	0			
	1st metaphase		5 (2)	22 (9)	59 (18)	59 (18)	78 (20)	50 (15)			
	+ 1 sperm			44 (19)	37 (12)	1 (0.3)	2 (0.5)	3 (1)			
	+ > 1 sperm			2 (1)	1 (0.3)	0	1 (0.3)	1 (0.3)			
Secondary oocyte	+ 1 sperm				103 (32)	4 (1)	2 (0.5)	0			
	+ > 1 sperm				1 (0.3)	2 (0.5)	0	0			
Ootid	+ 1 sperm					114 (34)	48 (13)	8 (2)			
	+ > 1 sperm					2 (0.5)	2 (0.5)	1 (0.3)			
2-cell							101 (26)	99 (30)			
3-cell								7 (2)			
Total eggs		202	207	237	319	331	383	328	2007	—	—
Total penetrated eggs		—	—	132 (56)	205 (64)	212 (64)	239 (62)	194 (59)	—	982	—

Figures in parenthesis are percentages.

* Eggs recovered before semination were not included in the calculation of these percentages. Cleaved eggs were not included in the calculation of the incidence of polyspermy for the lower group (4%).

80 minutes. The authors just cited asserted also that pronuclear fusion took place at about 40 minutes; in the present series, the early prophase changes of the first cleavage division were observed between 50 and 60 minutes after semination. The implication is that fertilization was slower in the present series, probably owing to differences in water temperature (24° C. in the study of Costello *et al.*, 18° C. in the present series).

Two features having temporal association with the breakdown of the germinal vesicle are worthy of comment, and they may be attributable to the same underlying cause. These features are the large differences in the incidence of polyspermy before (24%) and after (4%) germinal vesicle disappearance, and the difference in the fate of penetrating sperm heads before and after the event. The difference in the incidence of polyspermy was the more striking since eggs with intact germinal vesicle showed a lower frequency of sperm penetration (54%) than did those undergoing maturation (67%). The deficiency in the former group presumably lay in the block to polyspermy, and the inference is that, when maturation begins, the nature of the reaction shown by the egg plasma membrane to sperm contact undergoes an important change. Normally, sperm penetration involves early fusion between egg and sperm plasma membranes (Colwin and Colwin, 1960; Szollosi and Ris, 1960; Friedmann, 1962), and it may well be that the block to polyspermy is provoked by such fusion. However, in oocytes before maturation, sperm entry may possibly occur in the way it was classically supposed to happen, namely, by a process resembling phagocytosis. With a phagocytic form of sperm absorption, the spermatozoon would be engulfed with its membrane intact and surrounded at a short distance by an envelope of egg plasma membrane. Early stages of what could be phagocytic engulfment of a sperm by a *Lytechinus* primary oocyte have been described by Franklin and Metz (1962). Under these circumstances, the sperm head would be shielded from cytoplasmic agents normally responsible for bringing about its metamorphosis into a male pronucleus. Thus, the high incidence of polyspermy and the persistence of unchanged sperm heads in the oocyte before breakdown of the germinal vesicle can both be explained by the assertion that the reactivity of the egg plasma membrane to sperm contact is not yet of the kind needed for normal fertilization.

The fluorescent colors displayed by oocytes treated with acridine orange and ultraviolet radiation conform in general to the descriptions given by Tweedell (1959, 1960, 1962). They are difficult to interpret. In fixed mammalian tissue subjected to the same treatment, yellow and red fluorescence was shown to be associated with the presence of DNA and RNA, respectively (Armstrong, 1956). Living mammalian eggs were found to have strongly green and red fluorescent structures; here the green color was regularly evident in DNA-containing elements but the distribution of the red color was consistent with an association, not with RNA, but with mononucleotides (Austin and Bishop, 1959). In *Pectinaria,* the red fluorescence could reasonably be ascribed to the presence of mononucleotides, but the cytoplasmic green fluorescence seems most unlikely to be indicative of DNA. The cytoplasmic DNA of *Paracentrotus* eggs is not demonstrable by histochemical methods, owing apparently to its low order of polymerization (Hoff-Jørgensen, 1954), and the acridine-orange-induced green fluorescence in the cytoplasm of *Arbacia* oocytes was not removed by DNAase (H. Esper, 1962, personal communication). Possibly, green fluorescence in these marine eggs can be ascribed to component proteins having an appropriate degree of polymerization.

The work described in this report was done when the author, a member of the External Scientific Staff, Medical Research Council, London, was F. R. Lillie Memorial Fellow for 1961 at the Marine Biological Laboratory, Woods Hole, Massachusetts. The observations on induced fluorescence were made in collaboration with Dr. C. B. Metz who also provided the fluorescence microscope and other facilities required for this procedure.

SUMMARY

1. The eggs shed by *Pectinaria gouldii* were in the stage of primary oocytes with intact germinal vesicles. Maturation began promptly, but proceeded only to the first metaphase, which some eggs reached in 20 minutes, and further progress depended upon sperm penetration.

2. Spermatozoa entered eggs rather more readily after than before breakdown of the germinal vesicle, and only those that entered after the event developed into pronuclei. It is suggested that spermatozoa entering eggs before maturation may be engulfed in a manner resembling phagocytosis, as distinct from membrane fusion. Polar bodies were extruded about 20–25 minutes and 30–35 minutes after semination at 18° C. Male pronuclei were evidently larger than the female. Early prophase of the first cleavage mitosis was seen between 50 and 60 minutes after semination, and cell division took place 20 to 30 minutes later at 18° C.

3. The incidence of polyspermy observed was 24% before and 4% after breakdown of the germinal vesicle. Eggs undergoing refertilization, possible early gynogenesis, and development after failure of polar-body formation were also seen.

4. Oocytes treated with acridine orange displayed green and red cytoplasmic granules, as well as a green zone around the nucleolus. The red fluorescence may have denoted the presence of mononucleotides; the green fluorescence was not considered specific to DNA.

LITERATURE CITED

Armstrong, J. A., 1956. Histochemical differentiation of nucleic acids by means of induced fluorescence. *Exp. Cell Research,* **11**: 640–643.

Austin, C. R., and M. W. H. Bishop, 1959. Differential fluorescence in living rat eggs treated with acridine orange. *Exp. Cell Research,* **17**: 35–43.

Colwin, A. L., and L. H. Colwin, 1960. Changes in the spermatozoon during fertilization in *Hydroides hexagonus* (Annelida). II. Incorporation with the egg. *J. Biophys. Biochem. Cytol.,* **10**: 255–274.

Costello, D. P., M. E. Davidson, A. Eggers, M. H. Fox and C. Henley, 1957. Methods for Obtaining and Handling Marine Eggs and Embryos. Marine Biological Laboratory, Woods Hole, Massachusetts.

Franklin, L. E., and C. B. Metz, 1962. Electron microscope study of sperm entry into sea urchin oocytes. *Biol. Bull.,* **123**: 473.

Friedmann, I., 1962. Cell membrane fusion and the fertilization mechanism in plants and animals. *Science,* **136**: 711–712.

Hoff-Jørgensen, E., 1954. Deoxynucleic acid in some gametes and embryos. *In:* Recent Developments in Cell Physiology, p. 79. Ed. by J. A. Kitching. Butterworths Scientific Publications, London.

Szollosi, D. G., and H. Ris, 1960. Observations on sperm penetration in the rat. *J. Biophys. Biochem. Cytol.,* **10**: 275–283.

Tweedell, K. S., 1959. Induced fluorescence in marine eggs. *Biol. Bull.,* **117**: 429–430.

Tweedell, K. S., 1960. Differential staining of cytoplasmic inclusions in eggs of *Pectinaria. Biol. Bull.,* **119**: 345.

Tweedell, K. S., 1961. Factors affecting germinal vesicle breakdown in *Pectinaria (Cistenides) gouldii. Biol. Bull.,* **121**: 412.

Tweedell, K. S., 1962. Cytological studies during germinal vesicle breakdown of *Pectinaria gouldii* with vital dyes, centrifugation and fluorescence microscopy. *Biol. Bull.,* **123**: 424–449.

Wilson, D. P., 1936. Notes on the early stages of two polychaetes, *Nephthys hombergi* Lamarck and *Pectinaria koreni* Malmgren. *J. Mar. Biol. Assoc.,* **21**: 305–310.

MARINE BIOLOGICAL LABORATORY LIBRARY WOODS HOLE, MASS.

THE RELATION BETWEEN INTENSITY OF INDUCTOR AND TYPE OF CELLULAR DIFFERENTIATION OF RANA PIPIENS PRESUMPTIVE EPIDERMIS[1, 2]

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In a recent paper (Barth and Barth, 1962), we suggested that lithium chloride applied in various concentrations might be expected to induce the presumptive epidermis to differentiate into various cell types. Relatively low concentrations of lithium chloride regularly induced nerve, higher concentrations, pigment cells and, occasionally, very high concentrations for long periods induced muscle cells. The present investigations are concerned with a more detailed study of the effects of lithium chloride upon the presumptive epidermis of *Rana pipiens* gastrula.

EXPERIMENTAL PROCEDURE

An important departure from the methods used in previous experiments is the omission of agar as a substrate during the period of reaggregation of the partially dissociated cells of the presumptive epidermis. The aggregates are now prepared on the glass surface of a medium-sized stender dish and transferred at desired intervals to the small-sized stender dishes used as culture dishes. The use of a glass surface rather than agar became desirable when it was found that agar has a neuralizing effect on presumptive epidermis. This effect was first found in experiments which called for a short period during which the aggregates remained on agar before being transferred to the glass-bottom culture dishes. If the contact between the aggregate and agar was less than 30 minutes, the aggregates differentiated into a sheet of epithelium with ciliated patches instead of radial nerve with oriented cells. Aggregates exposed to agar from 45 minutes to 21 hours regularly differentiated with some type of nerve cells present.

The evidence for the neuralizing effect of agar is presented in Tables I and II. The first experiment compares the cellular differentiation obtained after the aggregates have been exposed to glass, to agar, and to glass surrounded by agar for 5 hours. The "glass surrounded by agar" was achieved by covering the bottom of the dish with agar in the usual manner and then removing strips of agar and placing the aggregates on the glass surface so exposed.

When no agar is present in the dish the aggregates upon transfer to culture dishes spread out into a thin sheet of epithelium with ciliated patches. Only one aggregate formed a few nerve fibers. In contrast the aggregates which had been exposed to agar, with or without direct contact, differentiated into spreading

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[2] The authors acknowledge with gratitude the valuable assistance of Iris Nelson.

TABLE I

The neuralizing effect of agar upon presumptive epidermis

Stage 11 gastrulae. Operating and culture medium: solution with 1 mg./ml. globulin (Bios). Agar: washed repeatedly in distilled water over prolonged period at 3–5° C. Dissolved in standard solution. Conditions: operation, dissociation in Versene and preparation of aggregates carried out on agar or glass as indicated and aggregates left for period indicated before transfer to small culture dishes without agar.

Exp. No.	Conditions	Time, hrs.	No. of aggregates	Cellular differentiation
1	Glass surface	5	30	Epithelium
	Agar surface	5	30	Spreading nerve
	Glass surface, agar sides	5	45	Spreading nerve
2	Agar film	7	40	Epithelium
		23	35	Epithelium
3	Agar, pH 6.9	6–8	75	Radial nerve
	Agar, pH 8.3	6–8	75	Radial nerve
	Agar, pH 7.0	0.5	35	Epithelium
	Agar, pH 7.0	2.5	40	Radial nerve
	Agar, pH 7.0	6.0	35	Radial nerve

nerve which is characterized by a network of nerve fibers and very few epithelial cells.

The neuralizing effect of agar is thus not the result of the type of surface provided by agar but is probably brought about by a diffusible substance present in the agar. Repeated washing with cold distilled water over prolonged periods

TABLE II

Effect of nitrogen and oxygen on the neuralizing action of agar

Time: time in hours of exposure to agar and gas

Time, hrs.	N_2		O_2		Air	
	No.	Diff.	No.	Diff.	No.	Diff.
0.1	40	Epithelium	30	Ciliated masses	40	Epithelium
0.5	85	Epithelium	30	Epithelium	40	Epithelium
0.75	35	Epithelium	50	Short nerve	40	Short nerve
1.0	50	Epithelium	40	Short nerve	35	Short nerve
1.5	115	Epithelium	75	Radial nerve	35	Radial nerve
1.75					40	Radial nerve
2.25					35	Short nerve
3.0	45	Epithelium Radial nerve			40	Short nerve
3.3	35	Radial nerve				
4.0			35	Radial nerve		
5.0			15	Radial nerve	40	Spreading nerve
6.0					35	Radial nerve
7.0					40	Spreading nerve
7.75	75	Spreading nerve Radial nerve			40	Spreading nerve
8.0	25	Spreading nerve	35	Spreading nerve		
21.0					35	Radial nerve

did not remove this substance. Since streptomycin sulfate antagonizes the neural differentiation produced by agar, we tried washing the agar with this compound. Such treated agar still induced nerve, however.

The second experiment recorded in Table I deals with a special situation in which an extremely thin film of agar was obtained by pouring hot agar into a hot dish and immediately pouring out the excess. A continuous film of agar resulted upon cooling, and the aggregates exposed to such a film for 7 to 23 hours differentiated into epithelial sheets with no nerve present. We conclude that the agar surface is not the neuralizing agent.

The third experiment was prompted by the possibility that, in the preparation of the agar by boiling in our salt solution, the phosphates precipitated and the pH became elevated. Other experiments show that sodium bicarbonate added to the solution to bring the pH to 8.8 induces nerve. The data show that agar at pH 6.9, 8.3 and 7.0 induces nerve if the exposure is more than 30 minutes. Since our salt solution for culture of the cells is at pH 8.0–8.2 and does not induce nerve at these pH's, the neuralization by agar dissolved in the salt solution is not brought about by a change in pH.

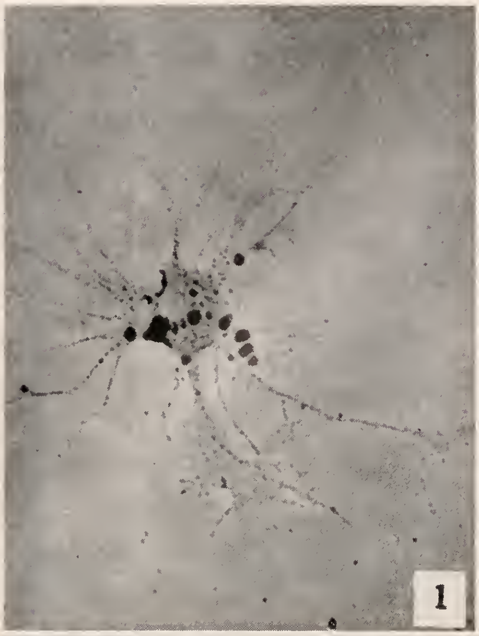
Table II gives additional evidence for the neuralizing effect of agar. Aggregates are permitted to form on agar and are left there for varying lengths of time. In air and in oxygen, 45 minutes' exposure is sufficient for neuralization. Further exposure results in more and more neural differentiation with fewer and fewer epithelial cells present. No significant differences in differentiation occurred in oxygen as compared with air. In nitrogen, however, epithelial sheets with little or no nerve were obtained for as long as 90 minutes. After 3.3 hours' exposure to agar and nitrogen, differentiation was predominantly nerve in character.

As a result of the experiments recorded in Tables I and II, we decided to carry out all the operations on glass for the experiments presented in this paper. Otherwise the method is the same as outlined in Barth and Barth (1962).

Classification of Cellular Differentiation

We will use the same designation of the types of differentiation obtained by treatment with various compounds as was used in Barth and Barth (1962). A brief description follows:

1. Epithelium: The cells spread out in a sheet of epithelial cells, patches of which become ciliated. A variant results when the aggregate fails to attach and free-swimming ciliated masses of cells result.
2. Radial nerve: Some of the cells spread out as an epithelial sheet while the cells in a centrally located mass send out nerve fibers over the surface of the epithelial sheet. Later the cells in the epithelial sheet become elongated and oriented in parallel fashion about the central mass of nerve cells. The epithelial cells do not form cilia.
3. Spreading nerve: Few epithelial cells are present and these few are scattered about the periphery of a diffuse nerve network. The latter is characterized by an extensive migration of neuroblasts to form several loci for formation of nerve fibers.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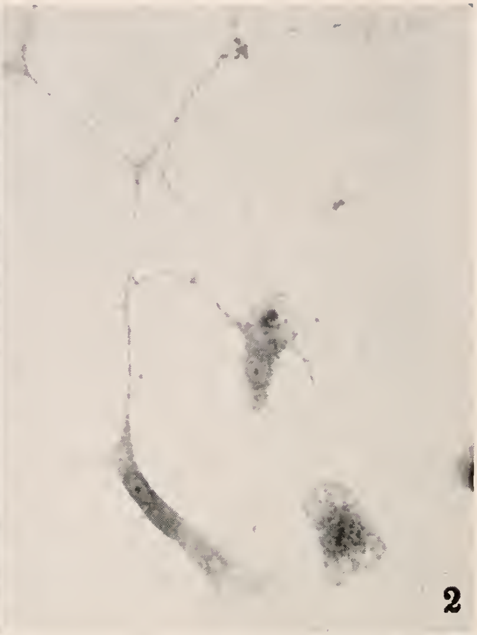
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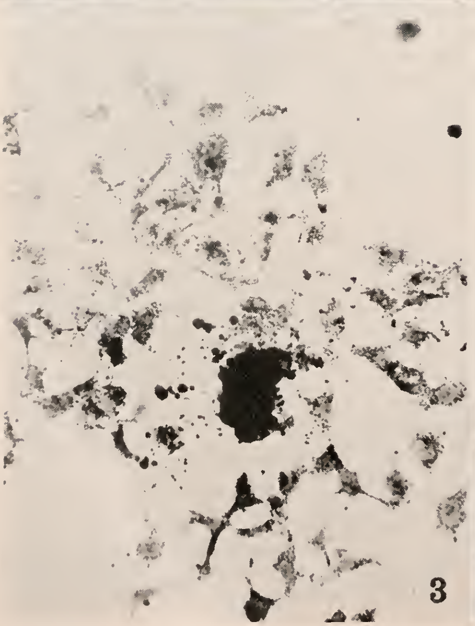
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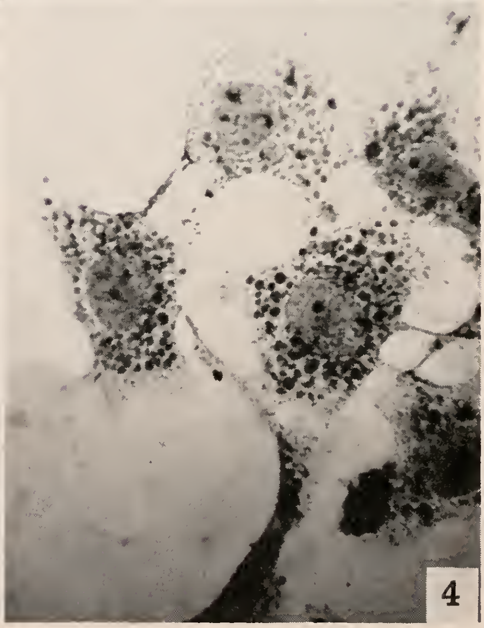
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FIGURE 1. An "astrocyte" from a culture prepared by treating presumptive epidermis cells of stage 11 gastrula with a solution of lithium chloride (0.74 mg./ml. of standard solution) for 47 hours at 23° C. On return to standard solution the cells differentiated as large pigment ring cells and transformed into "astrocytes." Fixed and stained after 37 days of culture at 23°–24° C.

4. Short nerve: No epithelial cells are present. The neuroblasts remain in a dense mass and send out numerous nerve fibers which turn and run parallel to the mass of neuroblasts.

5. Pigment cells: The original aggregate spreads out and the cells appear to repel each other so that single cells, approximately equidistant from each other, result. Most of these cells develop a ring of slate-gray pigment granules in contact with the nuclear membrane. These cells later form melanophores. Some mesenchyme cells also are present.

In addition three new types of cellular differentiation have been obtained.

6. "Astrocytes": Large star-shaped cells with a large pigment ring and filaments containing granules. These cells persist for three to four weeks and resemble astrocytes. Figure 2 shows such a cell with its filaments attached to adjacent cells. Figure 1 shows a typical "astrocyte."

7. "Neuroglia": Very large cells with long thick filaments filled with granules. They first arise as large single cells and as the filaments develop they connect with the filaments of adjacent cells. The result is a network or sometimes a lattice work of cell filaments. The cells resemble neuroglia cells. Figures 3 and 4 show these cells at 4 days while Figures 5 and 6 illustrate the connections between cells on the seventh day.

8. Calcium-induced nerve: This type of cellular differentiation is not described by either radial nerve or spreading nerve or short nerve. Although there are some epithelial cells and these become oriented as in radial nerve, there are also many nerve fibers growing out from the central mass into a periphery relatively free of epithelial cells. These fibers resemble short nerve. Then, too, although there is some migration of neuroblasts as in spreading nerve, most of the neuroblasts remain in a dense central mass. For purposes of identification we will use the term calcium-induced nerve to describe the above cultures (Figs. 7 and 8).

Although any one of these types of cellular differentiation may be induced with high frequency, in any one experiment, there is almost always some other type present. For example after certain treatments with lithium chloride most of the aggregates will form pigment ring cells but a few aggregates will also form nerve cells in addition to pigment ring cells. In the tables such a result would be classified as "pigment cells."

Similarly, our controls are now epithelial sheets with ciliated patches but a few aggregates in some experiments have formed some nerve. These are still classified as "epithelium."

In addition there are some inconsistencies in the tables. These are probably the result of one or all of three variables which are not accurately controlled. First, there is the stage of the donor gastrula. While we try to use stage 11 consistently, stage 11 itself is a variable and of course the age of the presumptive epidermis is important in relation to its competence to be induced. Second, we

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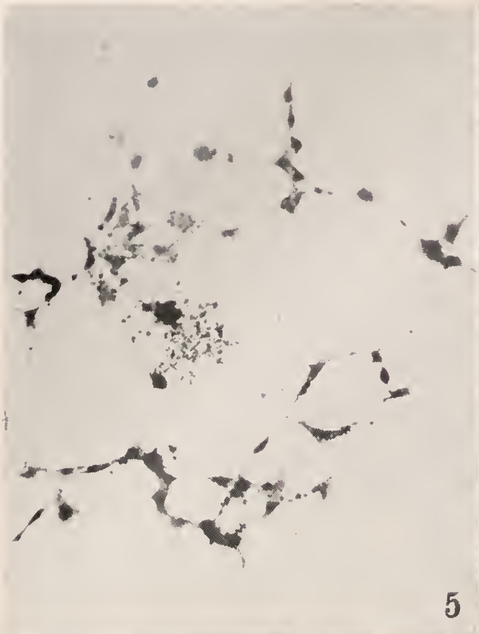
FIGURE 2. Same as Figure 1 and illustrates tendency of "astrocytes" to make contact with adjacent cells.

FIGURE 3. A 4-day culture of "neuroglia" cells, produced by culturing the presumptive epidermis of stage 11 gastrula in a solution containing 0.47 mg. of lithium chloride per ml. of standard solution. These cells contain no pigment rings.

FIGURE 4. Same as Figure 3 but higher magnification, showing absence of pigment rings and presence of yolk.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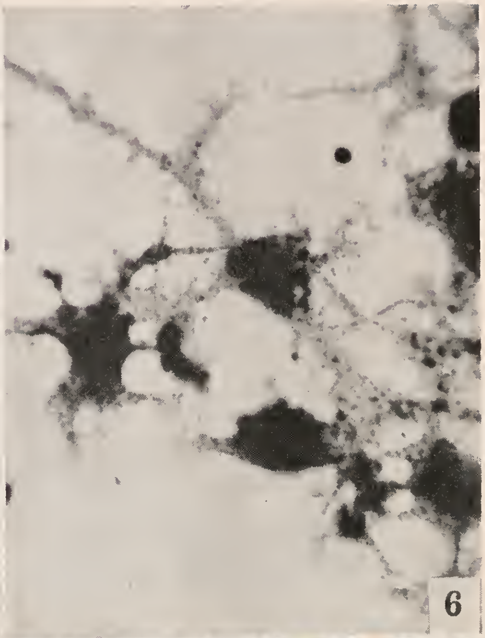
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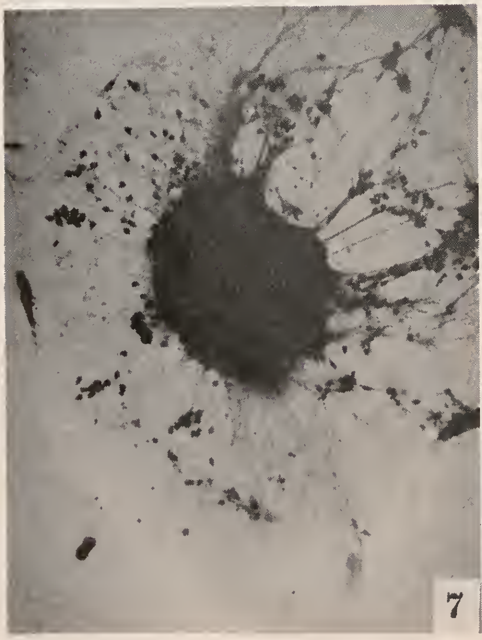
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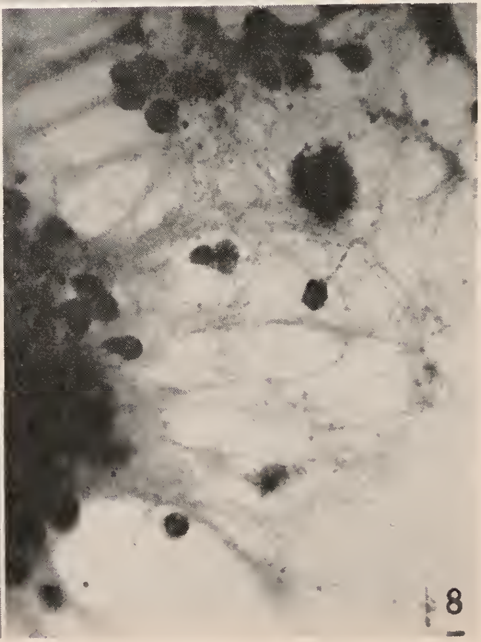
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FIGURE 5. A 7-day culture of "neuroglia" cells prepared by culturing the presumptive epidermis of stage 11 gastrula in a solution containing 0.56 mg. of lithium chloride per ml. of standard solution. Most of the cells are connected with each other by protoplasmic filaments.

FIGURE 6. A detail of Figure 5, showing the granular nature of the protoplasmic filaments.

have not accurately controlled the temperature during the treatment of the aggregates. We use room temperature which is controlled by air conditioners. In early experiments we found the vibration of constant temperature incubators to have a deleterious effect on the attachment of the aggregates.

Finally the size of the aggregate we use varies and size may be important in determining the concentration of the test solution at the cell surfaces. For example, lithium chloride in low concentrations induces nerve cells while in higher concentrations pigment cells form. In a relatively large aggregate in a high concentration of lithium we find that the peripheral cells form pigment cells while the centrally located cells become nerve cells. Is this a result of the relatively large mass having been subjected to a gradient of concentrations of lithium, high at the periphery and low in the center?

RESULTS

We will first report the results of experiments designed to test whether inductors such as sodium bicarbonate, magnesium sulfate, ammonium sulfate and calcium chloride induce the presumptive epidermis to form various types of nerve when the neuralizing effect of agar is eliminated. Second, the results of an extensive series of experiments in which lithium chloride in various concentrations is used as an inductor for varying lengths of time will be presented.

Sodium bicarbonate. The addition of 1 mg./ml. of $NaHCO_3$ induces the presumptive epidermis to form nerve cells. Table III, Exp. 1, records the results of exposing the aggregates for periods varying from 0.1 hour to continuous exposure. Actually some of the aggregates have been exposed longer than the recorded time since it takes about 20 minutes to prepare them. This probably accounts for the little nerve present with a short exposure of 0.1 hour. Typically, however, a short exposure results in extensive sheets of epithelial cells, some of which are ciliated. Nine hours' treatment induces extensive radial nerve together with elongate cells oriented in parallel fashion. Some of the aggregates form short nerve with no epithelial cells present. With longer exposure or continuous exposure the aggregates exhibit no migration of epithelial cells but remain as a dense mass of cells. Nerve fibers develop slowly and turn to run parallel to the circumference of the mass of neuroblasts. Many fibers form a dense intricate pattern of fibers classified as short nerve.

Exp. 2 confirms the first experiment and was designed to test the period of competence of the presumptive epidermis for neuralization by sodium bicarbonate. Again a short exposure results in a little nerve while the control shows no nerve. The competence after 0.5 hour remains unaltered and short nerve develops in sodium bicarbonate. After 7 hours' treatment with sodium bicarbonate the aggregates form short nerve in our standard salt solution. If, however, the aggregates are prepared in a standard salt solution and remain in it for 7 hours and then are transferred to sodium bicarbonate, little or no nerve develops and the cultures resemble controls very closely. Both sets of cultures contain pre-

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FIGURE 7. Calcium-induced nerve produced by culturing the presumptive epidermis of stage 11 gastrula in a solution containing 0.54 mg. $CaCl_2 \cdot 2H_2O$ per ml. of standard solution. Fixed at 7 days.

FIGURE 8. A detail of Figure 7, showing high frequency of nerve fibers.

dominantly epithelium. Thus, stage 11 presumptive epidermis loses its competence to react to added sodium bicarbonate in 7 hours at 24° C. By this time the whole gastrula controls are in stage 12.

Exp. 3 records the effects of a lower concentration of sodium bicarbonate. A sojourn of 0.5 hour in a concentration of 0.95 mg./ml. has no effect and the presumptive epidermis does not form nerve. If the aggregates remain in standard salt solution for 0.5 hour and then are cultured in 0.95 mg./ml., nerve is induced. Competence decreases after 7 hours at 19° C. and very little nerve is induced by added sodium bicarbonate.

TABLE III

Effects of sodium bicarbonate on the differentiation of presumptive epidermal cells, stage 11

The normal concentration of $NaHCO_3$ in our standard solution is 0.2 mg./ml. In this and the succeeding tables the table headings are to be interpreted as follows. Stage: Shumway (1940); Treatment, conc.: the final concentration of the test substance in milligrams per milliliter of standard solution; Time: the length of time in hours during which the aggregates are exposed to the test solution; Culture conc.: the concentration of the test substance in milligrams per milliliter of the standard solution to which the aggregates are transferred; No.: the number of aggregates treated; Types of cellular differentiation: as defined in text.

Exp. No.	Treatment				Culture			Results
	Conc., mg./ml.	pH	Time, hrs.	Temp., ° C.	Conc., mg./ml.	pH	No. aggregates	Types of cellular differentiation
1	1.2	8.9	0.1	21	0.2	8.0	30	Epithelium, little nerve
	1.2	8.9	9.0	21	0.2	8.0	35	Radial nerve, short nerve
	1.2	8.9	22.0	21–25	0.2	8.0	40	Short nerve
	1.2	8.9	0.5	24	1.2	8.9	25	Short nerve
2	1.2	8.9	0.5	24	0.2	8.2	25	Epithelium, little nerve
	0.2	8.2	0.5	24	0.2	8.2	25	Epithelium, no nerve
	0.2	8.2	0.5	24	1.2	8.9	25	Short nerve
	1.2	8.9	7.0	24	0.2	8.2	25	Radial nerve
	0.2	8.2	7.0	24	0.2	8.2	25	Epithelium
	0.2	8.2	7.0	24	1.2	8.9	25	Epithelium, little nerve
3	0.95	8.8	0.5	19	0.2	8.1	35	Epithelium, ciliated masses
	0.2	8.1	0.5	19	0.95	8.8	40	Short nerve
	0.95	8.8	7.0	19	0.2	8.1	40	Epithelium, some nerve
	0.2	8.1	7.0	19	0.95	8.8	35	Epithelium, little nerve

Magnesium sulfate. Table IV summarizes the effects of various concentrations of magnesium sulfate applied for varying lengths of time. While all concentrations used induce nerve if applied long enough, the higher concentrations induce spreading nerve while the lower concentrations induce radial nerve. Similarly, if the concentration is kept constant and the time varied, long exposures result in spreading nerve while with short exposures radial nerve develops. Thus, 6.2 mg./ml. of magnesium sulfate induces some radial nerve at 1.3 hours; after 4.3 hours all radial nerve is obtained, while after 5.5 hours the cultures are mostly spreading nerve.

It is of interest to point out that the presumptive epidermis survives continuous culture in concentrations as high as 5.2 mg./ml. This is a 25-fold increase in the concentration of magnesium sulfate used in the standard culture medium.

Ammonium sulfate. The effects of ammonium sulfate at two concentrations are given in Table V. At the lower concentration, 0.024 mg./ml., the presumptive epidermis survives 21 hours' exposure, but not continuous treatment. Three hours' treatment has no visible action and the presumptive epidermis spreads out as a sheet of epithelial cells. After 5 hours some radial nerve is formed, and after 8 and 21 hours' treatment all cultures exhibit radial nerve. Continuous exposure to 0.024 mg./ml. results in death.

TABLE IV

The effects of magnesium sulfate ($MgSO_4 \cdot 7H_2O$) on the differentiation of presumptive epidermis cells (stage 11)

Treatment			Culture		Results
Conc., mg./ml.	Time, hrs.	Temp., ° C.	Conc., mg./ml.	No. aggregates	Types of cellular differentiation
2.2	8.3	24	0.2	25	Radial nerve
2.2	0.5	24	2.2	25	Radial nerve
3.2	4.0	23	3.2	75	Radial nerve, spreading nerve
4.2	4.0	23	4.2	75	Spreading nerve, radial nerve
5.2	0.25	22	5.2	75	Spreading nerve
6.2	1.3	22	0.2	25	Epithelium, radial nerve
6.2	4.3	22	0.2	25	Radial nerve
6.2	5.5	22	0.2	25	Spreading nerve, radial nerve
6.2	1.5	23	0.2	35	Radial nerve
6.2	4.5	23	0.2	40	Radial nerve
8.2	2.5	24	0.2	35	Radial nerve
8.2	6.0	24	0.2	40	Spreading nerve
8.2	24.0	23.5	0.2	35	Spreading nerve
8.2	28.0	23.5	0.2	40	Spreading nerve
10.2	1.3	22	0.2	25	Spreading nerve, radial nerve
10.2	4.3	22	0.2	25	Spreading nerve
10.2	5.5	22	0.2	25	Spreading nerve
10.2	1.5	22	0.2	40	Radial nerve
10.2	4.5	23	0.2	35	Spreading nerve
15.2	2.0	22	0.2	35	Radial nerve
15.2	3.0	22	0.2	40	Radial nerve

An increase in tolerance to 0.024 mg./ml. of ammonium sulfate is shown by the results of transfers from standard solution to ammonium sulfate at various intervals. After 3 and 5 hours the presumptive epidermis begins to spread in 0.024 ammonium sulfate but soon dies, while after 8 and 21 hours epithelial sheets and ciliated masses form and persist in ammonium sulfate.

At a concentration of 0.25 mg./ml. ammonium sulfate induces radial nerve after 3 hours' exposure time, nerve after 5 hours and causes death after 7.5 hours' exposure time.

Table V

The effects of ammonium sulfate on differentiation of presumptive epidermis cells (stage 11)

Treatment			Culture		Results
Conc., mg./ml.	Time, hrs.	Temp., ° C.	Conc., mg./ml.	No. aggregates	
.024	3	20–21	0	25	Epithelium
.024	5	20–21	0	25	Epithelium, radial nerve
.024	8	20–21	0	25	Radial nerve
.024	21	20–21	0	30	Radial nerve
.024	3	20–21	.024	25	Dead
0	3	20–21	.024	25	Epithelium → dead
0	5	20–21	.024	25	Epithelium → dead
0	8	20–21	.024	25	Epithelium, ciliated masses
0	21	20–21	.024	15	Ciliated masses
.25	1.0	24.5	0	35	Epithelium
.25	3.0	24.5	0	35	Radial nerve
.25	5.0	24.5	0	40	Nerve
.25	7.5	24.5	0	35	Dead

Calcium chloride. Various nerve patterns are obtained when calcium chloride is added to our standard salt solution. As Table VI shows, the presumptive epidermis in the standard salt solution with 0.04 mg./ml. of calcium chloride forms chiefly extensive sheets of epithelial cells with cilated patches. Only a few cells in the center of the sheet form nerve fibers. With 0.15 mg./ml. of added calcium chloride radial nerve is predominant, and when 0.25 mg./ml. is added the presumptive epidermis forms chiefly spreading nerve with few epithelial cells.

Higher concentrations, 0.54 and 1.04, induce short nerve and a new nerve pattern which has been termed "Ca-induced nerve." This latter nerve pattern has some of the characteristics of "short nerve" and some of "spreading nerve."

Table VI

The effects of calcium chloride ($CaCl_2 \cdot 2H_2O$) on the differentiation of presumptive epidermis cells (stage 11)

Standard solution contains 0.04 mg./ml.

Treatment		Culture		Results
Conc., mg./ml.	Time, hrs.	Conc., mg./ml.	No. aggregates	
0.04	2.0	.04	75	Epithelium, little nerve
0.19	2.0	.19	75	Radial nerve
0.29	2.0	.29	75	Spreading nerve, radial nerve
0.54	7.0	.04	35	Short nerve
0.54	2.0	.54	35	Ca-induced nerve
0.54	1.5	.54	75	Ca-induced nerve
1.04	7.0	0.04	40	Short nerve
1.04	2.0	1.04	40	Ca-induced nerve
2.54	3.0	0.04	35	Radial nerve
2.54	5.5	0.04	40	Spreading nerve, short nerve

There are very few epithelial cells in the periphery and many short nerve fibers but also some long nerve fibers.

As with magnesium sulfate it is interesting to note that the cells tolerate continuously a 25-fold increase in the concentration of calcium chloride.

Lithium chloride. The data on the effects of lithium chloride are too extensive to report in detail. Preliminary experiments indicated that the type of cellular differentiation could be controlled either by the concentration of lithium chloride or by the time of exposure. These correlations are seen in Table VII, where various concentrations have been applied to the presumptive epidermis for varying lengths of time. For example at 1.5 mg./ml. a 10-minute exposure results in epithelium with ciliated patches as in controls. As the length of treatment is increased we first obtain radial nerve, then spreading nerve and finally pigment ring cells.

TABLE VII

The effects of lithium chloride on the differentiation of presumptive epidermis cells (stage 11)

Conc., mg./ml.	Duration of treatment						
	10 min.	15–30 min.	1 hr.	2 hr.	4 hr.	5 hr.	6–9 hr.
0.50	Epithelium		Epithelium, nerve	Epithelium Radial nerve Pigment			Pigment
0.75			Nerve, epithelium		Pigment, nerve Epithelium		
1.00	Epithelium	Epithelium	Radial nerve	Spreading nerve Radial nerve	Pigment	Pigment	
1.25			Radial nerve	Spreading nerve	Pigment, nerve	Pigment	
1.50	Epithelium	Epithelium Radial nerve	Radial nerve Spreading nerve	Pigment Spreading nerve	Pigment, nerve	Pigment	Pigment
2.00	Epithelium		Pigment, nerve	Pigment	Pigment	Pigment	Pigment
2.50					Pigment		
3.00			Pigment, nerve	Pigment	Pigment	Pigment	
3.50		Nerve	Pigment Radial nerve				
4.00		Radial nerve	Pigment, nerve	Pigment	Pigment		
6.00			Pigment, nerve	Pigment, nerve			

Similarly if we use one-hour exposure then with a concentration of 0.5 mg./ml. we obtain mostly epithelium with ciliated patches as in controls. Increasing the concentration to 1.0 mg./ml. results in radial nerve. With 1.5 mg./ml. we begin to find spreading nerve, and with still higher concentrations we obtain pigment ring cells.

Often two types of cellular differentiation are present in the same culture dish. This variability may be in part a result of the experimental procedure. The aggregates are prepared in lithium chloride and the time of exposure is measured from the time at which all the aggregates are prepared to the time of transfer to standard salt solution. Since it takes 20–25 minutes to prepare the 150 aggregates usually used for an experiment, some aggregates will have been exposed for as much as 25 more minutes than others. Of course the excised explant from which the aggregates are prepared has been in lithium chloride for the 20–25 minutes required for preparation of the aggregates and this fact may possibly minimize the difference in times of exposure of the aggregates.

During the course of the preceding experiments some of the aggregates were permitted to remain in lithium chloride continuously. While such treatment usually resulted in early death of the cells, the lower concentrations, 0.56 mg./ml., sustained the cells for about three weeks. These cultures did not develop nerve nor pigment ring cells but rather contained very large scattered cells which formed thick filaments containing granules (Figs. 4, 6). The filaments of adjacent cells made contact, forming a complex lattice work or network (Figs 3, 5). These cells are tentatively identified as neuroglia cells.

Table VIII records the effects of continuous treatment of presumptive epidermis with concentrations of lithium chloride ranging from 0.10 mg./ml. to 2.00 mg./ml. The "neuroglia" type cell is obtained at concentrations of 0.56 and 0.65 mg./ml. A 7-hour exposure to these concentrations gives the expected results, namely, pigment ring cells and nerve as well as some epithelium.

TABLE VIII

The effects of lithium chloride on the differentiation of presumptive epidermis cells (stage 11)

Conc. mg./ml.	Treatment	Results
0.10	continuous	Epithelium with ciliated patches; mucus cells
0.20	continuous	Ciliated masses secreting large amount of mucus; some epithelial sheets with ciliated patches
0.30	continuous	Ciliated masses and some epithelial sheets; mucus secreted
0.38	continuous	Ciliated masses with voluminous secretion of mucus containing pigment granules; astrocytes
0.47	continuous	Some ciliated masses with mucus; others form large scattered cells with protoplasmic filaments; neuroglia
0.56	continuous	All neuroglia
	7 hrs.	Epithelium, scattered pigment ring cells, little nerve
0.65	continuous	All neuroglia
	7 hrs.	Epithelium, pigment ring cells, nerve
0.74	continuous	Many loose cells, about 8–10 large neuroglia cells
0.91	continuous	Many loose cells, about 5–6 large neuroglia cells
1.50	continuous	Dead
	4 hrs.	Pigment, nerve
2.00	continuous	Dead
	4 hrs.	Pigment, nerve

We next attempted to see if there were any other cell types between the small pigment ring cells obtained with 6–9 hours treatment with low concentrations of lithium and the "neuroglia" cells found during continuous treatment.

Table IX reveals a new cell type resembling an astrocyte. These cells arise as large scattered cells similar to "neuroglia" but possess a large ring of pigment granules near the nucleus, as do the small pigment ring cells which give rise to melanophores. The "astrocytes," however, do not give rise to melanophores, and persist as star-shaped cells for about 4 weeks (Figs. 1, 2).

The sequence of inductions following a time course is shown by the data for 0.74 mg./ml. The epithelium is first induced to become a determined nerve cell. These determined nerve cells are then induced to become determined pigment cells. Further treatment induces the determined pigment cells to become determined "astrocytes" while continuous treatment induces the determined "astrocytes" to become "neuroglia" cells. These various cell types must be considered to be determined in the sense that placed in standard salt solution they self differentiate.

DISCUSSION

Induction by various ions

The induction of neural cells from the presumptive epidermis by various compounds is not a new phenomenon. Many substances of widely differing chemical constitution have been shown to be neural inductors. In some instances where the substances have been implanted the action has been assigned to a toxic or sub-cytolytic action. In other cases, where the presumptive epidermis is immersed in a solution of the substances, as in a conditioned medium (Niu and Twitty, 1953), it is difficult to accept a toxic action since the cells survive continuous exposure. There is of course the possibility that some cells are killed by the added substances and that the induction is actually brought about by the dead or injured cells. This latter possibility is always present since there is no way of obtaining presumptive epidermis without some injury caused by manipulation or operation. All we can do is prepare adequate controls for injury.

TABLE IX

The effects of lithium chloride on the differentiation of presumptive epidermis cells (stage 11)

Conc., mg./ml.	Time, hrs.	Results
0.47	19.5	Pigment cells, epithelium
	25.5	Pigment cells, epithelium
	continuous	Neuroglia
0.56	5.5	Epithelial cells, pigment cells
	20.5	Pigment cells
0.74	6.0	Small pigment ring → pigment cells
	21.0	Large pigment ring → astrocytes
0.74	5.5	Epithelium, few pigment cells, little nerve
	28.0	All pigment cells
	47.0	Large pigment ring → astrocytes
	continuous	Neuroglia
0.91	5.5	Pigment cells, nerve, epithelium
	28.0	Large pigment ring → astrocytes
	47.0	Large pigment ring → astrocytes
	continuous	Neuroglia
0.91	29.0	Pigment cells, astrocytes
	46.5	Astrocytes
	56.5	Astrocytes
1.50	29.0	Many dead, a few astrocytes
	46.5	Many dead, a few astrocytes
	56.5	Many dead, a few astrocytes

Our controls consisting of presumptive epidermis from the inner layer of the ectoderm of stage 11 gastrula now differentiate into epithelial sheets with ciliated patches. Only rarely does nerve appear. The injury phenomenon is definitely present and in our cultures there are always some unattached dead cells which appear about two days after the preparation of the aggregates. These dead cells do not induce nerve, however, under the conditions of our experiment. Therefore it is probable that the induction by such ions as Mg^{++}, Ca^{++} and HCO_3^- is a result of the alteration of the living cells and not a secondary phenomenon caused by the presence of dead cells. The cells survive continuous treatment with these ions.

The significance of the experiments on induction with Mg^{++}, Ca^{++} and HCO_3^- resides in the fact that they are normally present in living inductor cells and a release of any or all of these ions during gastrulation would result in induction of a neural plate. On the other hand, the normal inductor may bring about the release of any or all of these ions within the ectoderm cells with which it comes in contact. With these widely differing interpretations of the action of Mg^{++}, Ca^{++} and HCO_3^- possible we do not feel that these studies throw any light on the naturally occurring inductors in the chorda mesoderm.

Sequential Induction by Lithium Chloride

While the lithium ion can be definitely excluded as a natural inductor the sequence of inductions obtained by varying the concentration of lithium chloride may be of significance in the interpretation of the action of a naturally occurring inductor regardless of its chemical constitution.

Let us use epi for epidermis; N_1 for radial nerve; N_2 for spreading nerve; P for small pigment ring cells; A for "astrocytes" and Ng for "neuroglia" cells. Then we may formulate a hypothesis of 6 different pathways from presumptive epidermis to the 6 cell types:

$$\begin{array}{cccccc} \multicolumn{6}{c}{\text{undifferentiated ectoderm cell}} \\ \downarrow & \downarrow & \downarrow & \downarrow & \downarrow & \downarrow \\ \text{epi} & N_1 & N_2 & P & A & NG \end{array}$$

The various potencies of the presumptive epidermis are shown here as diverse pathways. Lithium chloride in low concentration of 1.25 mg./ml. for one hour might be supposed to inhibit all pathways but that leading to N_1, *i.e.*, radial nerve. The cells are thus determined and will differentiate into nerve cells in standard solution. If, however, these determined nerve cells be exposed to lithium chloride for four hours the cells will differentiate into pigment cells (P) in standard solution. Thus, the pathway toward P was not destroyed by the one-hour treatment. Similarly, it can be shown that after P is determined, further treatment with lithium chloride results in A or Ng. Thus, lithium chloride does not appear to act by inhibiting all pathways but one, and the concept of diverse pathways is not applicable. Rather the pathway to P, for example, must pass through N_1 and N_2.

We meet with a similar inconsistency if the assumption is made that lithium chloride in low concentration stimulates the pathway leading to N_1 while a high concentration stimulates the pathway to P. For again we would assume that the other pathways are lost when N_1 is determined. But further treatment after N_1 is determined results in N_2, still further in P, and with very long times A results. Thus, the pathways do not appear to be divergent but rather they are probably arranged in some linear order.

The foregoing criticisms of the diverse-pathways concept leads to the formulation of a sequence of potencies. The potencies would be present as a chain of reactions with epi and Ng at the ends of the chain. Whether we begin with epi or end with it depends upon whether we choose stimulation or inhibition as the mechanism for the induction. For example: if

$$\text{epi} \xrightarrow{1} N_1 \xrightarrow{2} N_2 \xrightarrow{3} P \xrightarrow{4} A \xrightarrow{5} \text{Ng},$$

then Li_1 could stimulate at 1, higher concentrations or longer times would stimulate 2, 3, 4, 5 progressively. Thus, at any time the cells might be determined to form P but the possibility of A and Ng still exists. In this formulation cells may be determined to form N_1 without losing the potencies in the rest of the sequence.

On the other hand if we write the sequence

$$\text{Ng} \xrightarrow{5} A \xrightarrow{4} P \xrightarrow{3} N_2 \xrightarrow{2} N_1 \xrightarrow{1} \text{epi},$$

then we express a concept of a series of reactions leading to the formation of epidermis unless stopped at some step. Mild inhibition by lithium chloride would then block the reaction 1, thus determining N_1, while more inhibition would block at 1 and 2, resulting in N_2. Still more inhibition might be expected to block 1, 2, 3, etc. Such a formulation retains all the reactions determining P when 1 and 2 are blocked, resulting in the determination of N_2. There would probably be some time limit on the possibility of determining P after N_2 is determined since the formulation shows the reactions proceeding from left to right. Presumably, with steps 1 and 2 blocked all the products of the sequence of reactions would be used in the differentiation of N_2. However, early in the process N_2 may be determined so that it will self differentiate in absence of induction but P may still be determined by a longer treatment with lithium chloride. This latter statement is simply a restatement of the results of the experiments reported in this paper.

Much of the research dealing with induction of the amphibian presumptive epidermis is difficult to interpret in light of the naturally occurring inductor or inductors. The present study is no exception. At first sight it is tempting to draw the parallel between the varying times of action of the organizer as gastrulation proceeds and the effects of lithium chloride at varying times of exposure to the presumptive epidermis. However, Mangold's transplantation of different regions of the roof of the archenteron to the blastocoele has shown that head induction and trunk induction may be obtained when the times of action are the same (Mangold, 1933). Thus, while it is definitely true that the posterior presumptive neural plate is exposed to the invaginating roof of the archenteron several hours before the anterior presumptive neural plate, this fact seems to be irrelevant to the induction process. The posterior neural plate may be induced by the posterior roof archenteron without previous contact by the anterior roof of archenteron.

It is true, however, that in the process of gastrulation the anterior roof of the archenteron first induces the presumptive spinal cord to become determined as forebrain. This determined forebrain then becomes determined as hindbrain under the influence of hindbrain inductor, and finally the determined hindbrain is induced to become spinal cord. Thus, the preliminary induction of forebrain does not exclude the later induction of spinal cord and in this respect normal induction resembles sequential induction by lithium chloride.

It is also tempting to conclude that, since different concentrations of lithium chloride induced different cell types, the organizer is merely a graded series of concentrations of a single inductor substance. And indeed there is supporting evidence from the report by Yamada (1958) on the changes of inductive ability of the bone marrow upon heating. Possibly also the fact that denaturation of the kidney pentose nucleo protein inductor changes its inductive power from posterior neural induction to anterior neural induction might be cited (Yamada, 1958).

On the other hand, the experiments with dead roof of archenteron show no differences in inductive ability between anterior and posterior regions. If different concentrations of some inductor were responsible for anterior and posterior neural plate, one would expect these to be present in the dead cells. For the dead cells are able to induce although they lost the regional specificity for induction. The situation becomes complex indeed when we begin to suggest that the induction by dead archenteron roof is not brought about by the natural inductor.

Perhaps the fairest statement would be since lithium chloride in various concentrations induces various cell types, it makes possible an interpretation of the organizer as a concentration gradient of a single inductor, but the experiments presented in this paper do not constitute direct evidence.

SUMMARY

1. Presumptive epidermis of stage 11 gastrula differentiates into epithelial sheets with ciliated patches.

2. Alteration of the basic salt solution by addition of calcium chloride or magnesium sulfate or sodium bicarbonate results in the differentiation of nerve cells.

3. Calcium chloride, magnesium sulfate, sodium bicarbonate, ammonium sulfate and lithium chloride will induce nerve cells from presumptive epidermis when applied for a few hours beginning with stage 11.

4. Lithium chloride applied to the presumptive epidermis for varying lengths of time induces various cell types.

5. Lithium chloride applied in various concentrations to the presumptive epidermis induces various cell types.

6. A concept of sequential induction is introduced as a formal explanation of the lithium chloride inductions.

LITERATURE CITED

BARTH, L. G., AND L. J. BARTH, 1962. Further investigations of the differentiation *in vitro* of presumptive epidermis cells of the *Rana pipiens* gastrula. *J. Morphol.*, **111**: 347–373.

MANGOLD, O., 1933. Über die Induktionsfähigkeit der verschiedenen Bezirke der Neurula von Urodelen. *Naturwiss.*, **43**: 761–766.

NIU, M. C., AND V. C. TWITTY, 1953. The differentiation of gastrula ectoderm in medium conditioned by axial mesoderm. *Proc. Nat. Acad. Sci.*, **39**: 985–989.

SHUMWAY, WALDO, 1940. Stages in the normal development of *Rana pipiens*. *Anat. Rec.*, **78**: 139–147.

YAMADA, T., 1958. Embyronic Induction. *In:* The Chemical Basis Of Development; pp. 217–218. Ed. by W. D. McElroy and B. Glass, Johns Hopkins Press, Baltimore, Md.

VARIATIONS IN THE LARVAL STAGES OF A DECAPOD CRUSTACEAN, PLEURONCODES PLANIPES STIMPSON (GALATHEIDAE)[1]

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During the last decade extensive literature has been accumulating on the larval development of several species of decapod Crustacea. Costlow, Bookhout and Monroe (1960) have reared several species of Brachyura. Rees (1959) has reared *Emerita talpoida,* Coffin (1958) successfully raised species of pagurids, and Broad (1957a and 1957b) raised two species of Macrura (*Palaemonetes*). The Brachyura, with rare exceptions, all pass through a constant number of molts, though duration of time spent in the various larval stages is influenced by temperature and salinity. Broad (1957b) found that the number of molts passed through, and the duration of the larval stages, in *Palaemonetes* were influenced by type of food available to the larvae. Among the Anomura the picture is less clear. Johnson and Lewis (1942), in a study of larvae of *Emerita analoga* from the plankton, described the morphology of several discrete stages, and reported several specimens which appeared intermediate between stage III and stage IV; the authors assumed that the intermediate forms indicated variation in the molting sequence. When Rees reared the larvae of *E. talpoida,* he noted that some went through a stage that was deleted by others. The precise molting history of individual larvae in many of the studies has been obscured in laboratory studies by the practice of rearing several larvae in the same container. In plankton studies the observers are generally unable to discern what degree of morphological variation occurs within the confines of the same larval stage.

In 1960 one of us (Boyd) described and figured five larval stages of *P. planipes,* based on specimens taken from the plankton in neritic waters off southern Baja California. The five stages were morphologically discrete, and it was assumed that each stage was passed through in a single molt. Since then the larvae have been reared through the larval stages, the immature phase, and on to maturity, a total span of about a year. Laboratory data support the validity of the five stages described and add a great deal of information concerning the larval development.

Pleuroncodes planipes is an anomuran galatheid crustacean, about 9 to 11 cm. long as adults, and resembling a small homarid lobster. These red crabs, as they are commonly called, exist both as pelagic and benthic animals. The crabs occur along the coasts of California and Baja California, over a range from 16° N. to 37° N. and have a distribution which is typically neritic in the pelagic phase. Distribution, growth rates and notes on the biology of the adults and post-larval crabs are presented elsewhere (Boyd, 1963).

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REPRODUCTION

Adult *Pleuroncodes planipes* females carry their eggs attached to their pleopods in the same manner as brachyuran and other anomuran crabs. Specimens in the laboratory bred and laid eggs readily; the eggs produced were carried from 6 to 22 days (generally about 14 days). During that time the eggs changed from a golden color to a dark amber, as the eyes of the embryos developed. All the larvae hatched out as swimming Stage I zoeae, and once hatching began, all the eggs carried by a female hatched within about 12 hours. Females carry up to 3650 eggs (the largest number counted in the study); larger females tend to have the greater number of eggs. Females kept isolated from males occasionally produced eggs, but these were invariably sterile, and were sloughed off from the pleopods within a few days. Records of individual females in the laboratory indicate that each female usually had two, and rarely three, broods of eggs per season. Sexual maturity, as denoted by the ability of the females to produce eggs, was attained at a size of 14–15 mm. standard carapace length. Females of that size are about 12 months old.

TABLE I

Numbers and ratios of post-larval male and female Pleuroncodes planipes caught in the monthly CalCOfI plankton samples, from December, 1958, to August, 1960. The number of these females which were carrying eggs is also given.

Cruise	Number & % males		Number & % females		Number & % of those females gravid		Month
5812	13	(54)	12	(46)	0	(0)	December
5901	293	(55)	241	(45)	27	(11)	January
5902	169	(51)	161	(49)	58	(36)	February
5903	16	(40)	24	(60)	0	(0)	March
5904	186	(58)	133	(42)	0	(0)	April
5905	111	(51)	106	(49)	0	(0)	May
5906	89	(52)	83	(48)	0	(0)	June
5907	193	(51)	186	(49)	0	(0)	July
5908	400	(60)	291	(40)	0	(0)	August
5909	46	(52)	42	(48)	0	(0)	September
5910	140	(55)	113	(45)	0	(0)	October
5911	3	(100)	0	(0)	0	(0)	November
5912	0	(0)	2	(100)	1	(50)	December
6001	199	(59)	141	(41)	41	(29)	January
6002	168	(61)	106	(39)	59	(56)	February
6003	324	(49)	334	(51)	133	(40)	March
6004	413	(55)	341	(45)	2	(.6)	April
6005	180	(46)	208	(54)	0	(0)	May
6006	71	(52)	66	(48)	0	(0)	June
6007	106	(57)	79	(43)	0	(0)	July
6008	87	(51)	84	(49)	0	(0)	August
Total	3207		2753				

In the laboratory the egg-bearing season lasted from November through April, with the peak in February. Table I shows that the egg-bearing season in the field followed a similar pattern.

The numbers of males and females caught in the plankton tows of the California Cooperative Oceanic Fisheries Investigations between December, 1958, and August, 1960, differed significantly from 50/50 at the 0.01 level, as tested by the signed ranks test, two-tailed. The average percentage observed was 53.85% males and 46.15% females. The reasons for this departure from the 50/50 sex ratio are not known, but the departure may result from one or more of the following: (1) more males may be hatched than females, (2) females may have a lower survival rate than males, or (3) the plankton nets may not sample males and females with equal effectiveness.

Methods

The rearing techniques used in this study are similar to those described by Broad, Coffin, Costlow *et al.*, and Rees. They involve the use of antibiotics to reduce the numbers of contaminating bacteria in the larval cultures, and also the use of *Artemia* nauplii for larval food. Freshly hatched larvae of *P. planipes,* taken from adult females kept in the laboratory, were pipetted into containers filled with sea water taken from the end of the pier of the Scripps Institution of Oceanography, La Jolla, California, where this work was done. The water had been filtered through glass wool to remove detritus and larger animals that might prey upon the larvae.

Experiment 1; started 19 February, 1960; duration 74 days. Two hundred freshly hatched larvae were placed, 10 per container, in 20 one-liter styrene plastic containers, each holding 500 ml. of water with antibiotics. The antibiotics used were (1) 50 mg./liter streptomycin (trade name "Combistrep" by Pfizer; dihydrostreptomycin and streptomycin sulfate; powder), and (2) 50 mg./liter penicillin (penicillin G by Abbott, pill form, buffered with $CaCO_3$, 928 units per mg., ground to a powder before use). This penicillin was used because earlier experiments indicated that the more readily available penicillin (a mix of 75% procaine penicillin and 25% penicillin G) was toxic to crab larvae. The containers were placed in trays of flowing sea water, which held them at temperatures ranging from 15° C. in February to 18° C. in June. Larvae were transferred to fresh sea water twice each week, and freshly hatched *Artemia* nauplii were added at that time. In the process of transferring the larvae into the fresh medium, each was drawn up into a 2-mm. bore glass pipette and examined through the pipette under a low power dissecting microscope to determine its stage.

Experiment 2; started 1 March, 1960; duration 74 days. One hundred larvae, 10 in each of 10 containers, were treated similarly to the larvae in Experiment 1, except that the penicillin was omitted; streptomycin was used in concentrations of 50 mg./liter; temperatures ranged from 15° to 18° C.

Experiment 3; started 12 April, 1960; duration 108 days. Ninety-three larvae were kept individually in plastic containers in 50 ml. sea water; 50 mg./liter streptomycin were added. Temperature, 16° to 19° C. Larvae were fed, transferred into new medium, and examined as in the preceding experiments. The containers were checked daily for exuviae and these, if present, were removed and preserved in glycerine on slides.

RESULTS OF EXPERIMENTS 1, 2, AND 3

The experiments proved that the larvae could be reared through all larval stages in the laboratory, and gave information concerning the total duration of the larval phase. The data are summarized in Table II.

Each of the seven larvae to complete the larval phase in Experiment 1, and one of the 56 in Experiment 2 passed through a new stage, VI. This stage has never been seen in a search of hundreds of larval specimens from the plankton. It was similar in morphology to Stage V, but was larger and characterized by a tuft of plumose setae on each of the pleopods. It is probable that it was an artifact of treatment, and its presence was due either to the penicillin or to the $CaCO_3$ buffer in the pills used, for other conditions were similar. As later experiments involving mixtures of non-buffered penicillin and streptomycin did not give Stage VI larvae, it is possible that the carbonate was the cause.

It was noted in the first two experiments that Stage IV had a longer duration than the other stages. Modal values in Experiment 2 were: Stage I, 9 days; Stage II, 8 days; Stage III, 8 days; Stage IV, 25 days; Stage V, 12 days. The mean durations could not be calculated because larvae were followed as a population, and not as individuals. It was suspected that Stage IV consisted of at least two sub-stages, but the exact number of sub-stages or the morphological differences between them could not be determined because the history of individual specimens could not be followed. Experiment 3 was set up to make it possible to follow individual specimens through the larval stages.

TABLE II

Duration and survival of larvae in Experiments 1, 2 and 3

Exp.	Shortest duration of larval phase	Longest duration of larval phase	Average	Survival through larval phase
1	54 days	68 days	61 days	7/200 = 3.5%
2	53 days	74 days	64 days	56/100 = 56%
3	71 days	110 days	87 days	15/93 = 16%

Although the volume of water per larva was identical (50 ml.) in each of the three experiments, and other conditions were the same in Experiments 2 and 3, the duration of the larval phase was longer in Experiment 3 than in either 1 or 2 (*cf.* data presented above). The three average values cannot be compared in the usual manner because the duration of the larval period in Experiments 1 and 2 is not known for individual larvae, and a variance cannot be calculated. A comparison of the ranges, however, indicates that the values for Experiment 3 differ significantly (at better than the 0.05 level) from the values for Experiments 1 and 2, but that the latter do not differ between themselves. The slightly greater temperature of Experiment 3, if it had any effect, should have caused a shortening of the larval period (see below). The only parameter in the three experiments which was known to be very different was the isolation of individual larvae in Experiment 3 versus their group rearing in Experiments 1 and 2. This difference had two components: the larvae had less total water in which to swim, though the volume

TABLE III

The percentage of larvae passing through each sub-stage of Stage IV (Experiment 3)

IVa = 100%
IVb = 100%
IVc = 100%
IVd = 100%
IVe = 82%
IVf = 65%
IVg = 59%
IVh = 29%
IVi = 2% (died before reaching Stage V)

of water per larva was identical, and they were not in association with other larvae. Either may have prolonged the larval phase in Experiment 3.

By following the molting sequence of individual larvae it became evident that the number of molts passed through before a larva completed the larval phase varied between larvae. The morphology of Stages I, II, and III in the laboratory was as described in 1960, based on larvae taken from plankton collections. Each of these stages was completed after a single molt. Stage IV, however, was divided into a series of what can be called sub-stages, each separated by a molt, but all morphologically within the general description of Stage IV. The larva increased in size through the sequence of sub-stages, and the number of setae on some of the appendages (*e.g.*, uropods, antennal scales) increased, but the differences between the various sub-stages of the complex were so inconsistent that a sub-stage could be identified with certainty only by knowing how many molts the larva had passed through. The number of sub-stages within the Stage IV complex varied from four to nine. The percentage of larvae passing through each sub-stage is given in Table III.

The mean duration of each of the larval stages and the 95% confidence limits of the sample, the cumulative elapsed time to the end of each particular stage (Σ), the number of larvae completing each stage (N), and the instantaneous death rate based on $N_t = N_o e^{-dt}$ are given in Table IV.

TABLE IV

The mean duration of each larval phase and the 95% confidence limits of the sample. The cumulative elapsed time to the end of each particular stage (Σ), and the number of larvae completing each stage (N)

Stage	Mean duration and 95% limits (days)	Σ (days)	N	Instantaneous death rate (days)
I	11.9 ± 5.7	11.9	46	0.059
II	8.0 ± 4.5	19.0	45	0.003
III	7.4 ± 5.7	27.3	41	0.013
IVa	6.9 ± 3.8	34.2	39	0.007
IVb	6.7 ± 5.9	40.9	36	0.012
IVc	7.4 ± 4.3	48.3	32	0.016
IVd	8.8 ± 5.4	57.1	28	0.015
IVe	8.7 ± 4.4	65.8	22	0.016
IVf	11.1 ± 6.6	76.9	18	
IVg	9.8 ± 4.7	86.7	16	
IVh	10.0 ± 4.0	96.7	8	
V	13.7 ± 6.6	86.9	15	0.000

The instantaneous death rate cannot be tabulated for individual stages subsequent to sub-stage IVd, for the deletion of stages is confused with mortality in the data analysis. It is evident from the table that the highest death rate occurred in the first stage, and mortality after that was essentially equal one stage to the next.

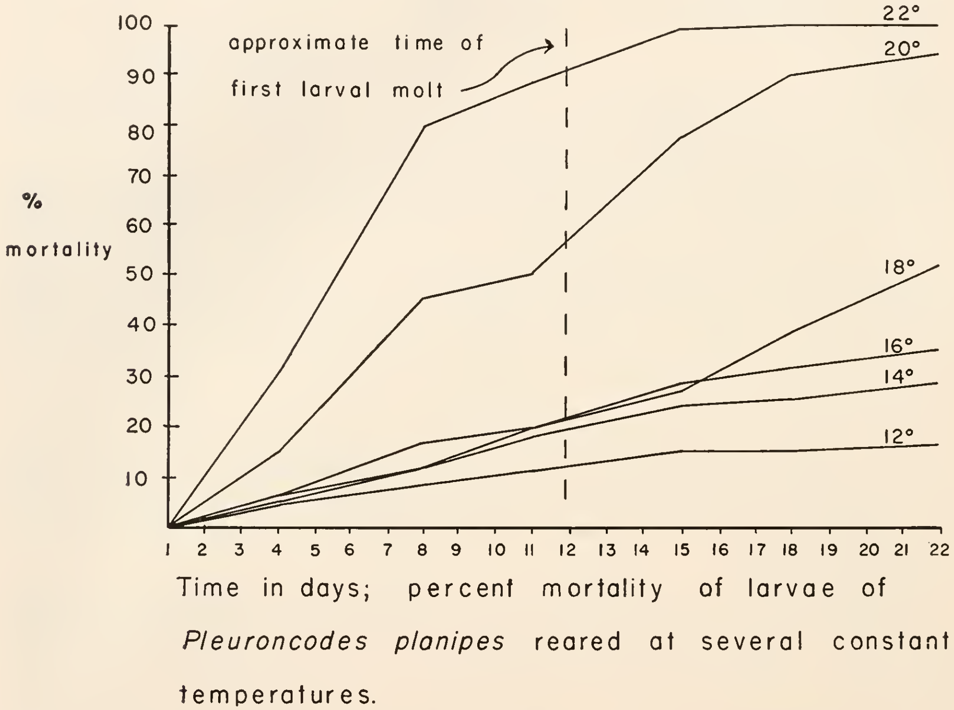

FIGURE 1. Mortality of larvae of *Pleuroncodes planipes* reared in the laboratory at several constant temperatures.

EXPERIMENT 4

METHODS

This experiment started April 25, 1960; duration 163 days. A device was designed and constructed which maintained larval cultures at six constant temperatures. The temperatures selected were 12°, 14°, 16°, 18°, 20°, and 22° C. The two extreme temperatures selected are approximately the surface temperatures of, respectively, the northern and southern ends of the distributional range of the adults in the spring months (the breeding season). Larvae were placed in sea water containing 50 mg./liter penicillin (Pfizer, penicillin G, potassium; 1585 units/mg. powder) and 50 mg./liter streptomycin (Combistrep, by Pfizer). Larvae were transferred into fresh sea water twice each week, and at that time were staged under the microscope and fed as in the previous experiments. The larvae were kept in styrene containers, each containing 18 compartments which measured 4.5 × 5.0 × 3.8 cm. deep and held 50 ml. of sea water. Six containers were used at each temperature; initially, two larvae were placed in each compartment, giving 216 larvae at each temperature, or a total of 1296 larvae. All of these larvae were obtained from the same female over a period of about 12 hours. The extra larva was placed in each compartment because Experiment 3 had shown that mortality was highest in the first few days. After 22 days the extra larvae in the 12°, 14°, and 16° cultures were discarded, leaving individual larvae (108 total) in the compartments; mortality had been higher at the higher temperatures, so that only 101 larvae remained at 18°, 16 larvae at 20°; and none at 22°.

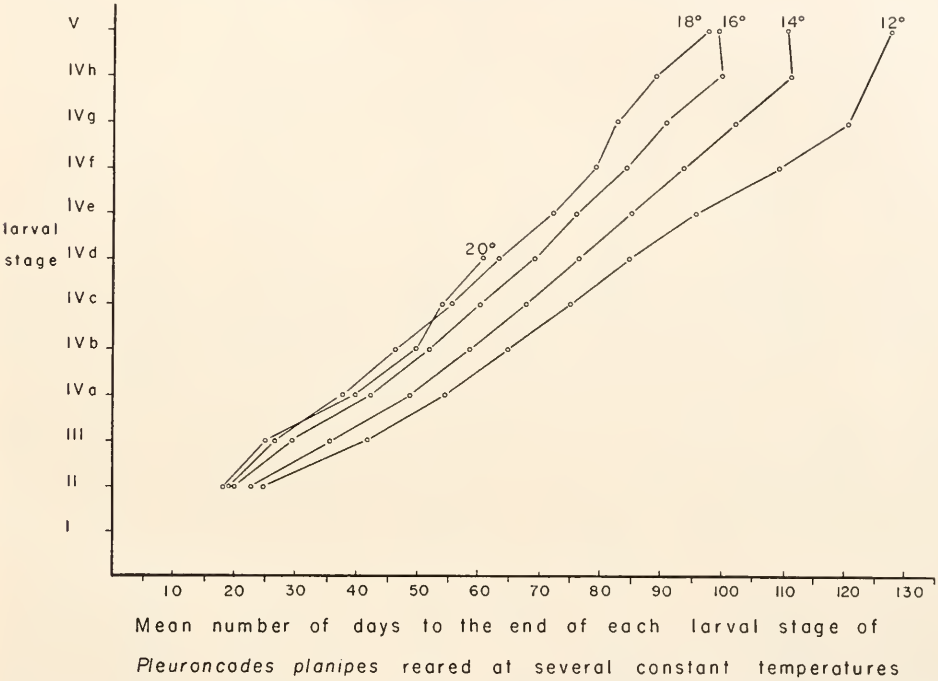

FIGURE 2. Rates of development of larvae of *Pleuroncodes planipes* reared in the laboratory at several constant temperatures.

RESULTS OF EXPERIMENT 4

The mortality data for the first 22 days are shown in Figure 1. The estimated instantaneous death rates through Stage 1 (0-11.9 days) are: 12°, 0.011; 14°, 0.018; 16°, 0.020; 18°, 0.020; 20°, 0.074; 22°, 0.192. No larvae completed the larval phase in either the 20° or 22° cultures; these temperatures may therefore be regarded as lethal in this experiment.

Within certain limits larval development should be faster at high temperatures; Figure 2 shows that this is true for *P. planipes,* at least over the range from 12° to 18° C. No data are available for the duration of Stage I because at the time the larvae were in that stage they were not kept individually. In Figure 2 it will be noted that the cumulative mean number of days to the end of sub-stage IVh is greater than the developmental time to the end of Stage V for the 14° and 16° cultures. This is because many larvae omitted one or more of the later Stage IV sub-stages and passed directly to Stage V, thereby shortening their larval duration. The trend of the 20° line is probably correct but it is based on too few individuals

TABLE V

Mean duration of stages of P. planipes larvae reared at several temperatures. These durations are cumulatively summed and presented under the heading Σ. The number of larvae passing through each stage is presented under N.

12°

Stage	Mean duration and 95% limits	Σ	*N*
II		24.7	
III	16.9 ± 7.0	41.6	84
IVa	12.8 ± 5.4	54.4	71
IVb	10.7 ± 5.8	65.1	68
IVc	10.4 ± 6.7	75.8	63
IVd	9.1 ± 5.1	84.6	60
IVe	11.7 ± 5.1	96.3	56
IVf	12.4 ± 7.0	108.7	50
IVg	12.0 ± 6.7	120.7	13
IVh			
V	17.7 ± 7.9	127.5	50

14°

Stage	Mean duration and 95% limits	Σ	*N*
II		22.3	
III	13.2 ± 8.1	35.5	61
IVa	12.6 ± 9.6	48.1	52
IVb	9.9 ± 5.9	58.0	45
IVc	9.5 ± 6.8	67.5	42
IVd	9.3 ± 5.2	76.8	37
IVe	7.2 ± 4.0	84.0	35
IVf	9.1 ± 3.8	93.1	33
IVg	8.9 ± 3.0	102.0	15
IVh	9.0 ± 5.7	111.0	2
V	13.2 ± 4.7	110.9	31

16°

Stage	Mean duration and 95% limits	Σ	*N*
II		19.7	
III	9.6 ± 7.1	29.3	57
IVa	12.8 ± 6.6	42.1	51
IVb	10.0 ± 6.9	52.1	43
IVc	8.8 ± 7.0	60.9	37
IVd	8.1 ± 5.8	69.0	34
IVe	7.6 ± 4.7	76.6	32
IVf	7.4 ± 5.9	84.0	28
IVg	7.2 ± 4.3	91.2	17
IVh	8.5 ± 4.3	99.7	2
V	10.6 ± 3.3	98.4	30

18°

Stage	Mean duration and 95% limits	Σ	*N*
II		19.0	
III	7.8 ± 6.0	26.8	33
IVa	10.6 ± 6.5	37.4	12
IVb	8.8 ± 4.8	46.8	9
IVc	9.2 ± 4.2	55.4	5
IVd	7.6 ± 2.8	63.0	5
IVe	9.0 ± 3.6	72.0	4
IVf	6.8 ± 1.1	78.8	4
IVg	4.0	82.8	2
IVh	6.0	88.8	1
V	11.7 ± 1.7	97.7	3

20°

Stage	Mean duration and 95% limits	Σ	*N*
II		18.0	
III	7.0	25.0	3
IVa	14.5 ± 3.5	39.5	2
IVb	10.0	49.5	1
IVc	4.0	53.5	1
IVd	7.0	60.5	1

TABLE VI

A. The number of larvae which molted directly to Stage V from each sub-stage, thereby omitting later sub-stages

B. The above data are expressed as the per cent of larvae which completed a particular sub-stage before becoming Stage V larvae

	12°	14°	16°	18°
IVe	3	1	3	0
IVf	37	17	11	1
IVg	13	13	15	1
IVh	0	2	2	1
V	53	33	31	3
IVe	100	100	100	100
IVf	94	97	90	100
IVg	24	45	55	67
IVh	0	6	6	33

to be very accurate; at the end of Stage III only three larvae were alive in the 20° culture. The mean duration of each stage (with the 95% confidence limits of the sample) is shown in Table V. The values are cumulatively summed to give the average total number of days elapsed to the end of each stage.

A Q_{10} value for the rate of larval development of *P. planipes* can be calculated on the basis of the mean number of days of life to the end of the larval phase. The first three values, 128, 111, and 98 (for 12°, 14°, and 16°, respectively), give an average Q_{10} of 1.95. The fourth value, for 18° (98), when compared with the 12° value gives a Q_{10} of 1.6. This figure is based on only three larvae at 18°, and is suspect; the correct value is probably about 1.9.

The number of sub-stages passed through in Stage IV varied from larva to larva in this experiment as it did in Experiment 3.

From Table V it can be seen that no larvae in the 12° culture went into sub-stage IVh; at other temperatures some larvae went through this sub-stage on their way to Stage V. The number of larvae which molted directly to Stage V from each sub-stage, and thereby omitted later sub-stages, is tabulated in Table VI (A). These data may be expressed as the per cent of larvae which completed a particular sub-stage before becoming Stage V larvae (Table VI (B)). A Friedman analysis of variance can be applied to the last three rows of figures in Table VI (B). This tests whether the columns of numbers come from the same population, and yields, in this case, a probability of 0.075. If one regards this value as significant, the data are evidence of a trend indicating that at higher temperatures larvae pass through more sub-stages than they do at lower temperatures. This is in spite of the fact that the total larval span in time is shorter at higher temperatures.

DISCUSSION

Perhaps the most interesting observation to result from the culturing of larvae of *P. planipes* in the laboratory is that Stage IV is divided into sub-stages, and that the number of these sub-stages may vary. The data indicate that the number of sub-stages which a larva passes through may be influenced by the temperature of the environment, with higher temperatures producing more sub-stages before the molt to Stage V; that within limits the rate of larval development shows a Q_{10} close to 2; and that higher temperatures cause a higher death rate.

Environmental factors other than temperature affect the duration of the larval stages. For example, temperatures were the same in Experiments 2 and 3, but there was a difference in the mean larval duration. The only known differences in conditions were the size of the rearing container and the presence of other larvae in the same container. Gurney (1942) conjectured that data concerning the life-histories of laboratory-reared larvae might prove misleading when applied to larvae in the ocean. In view of the demonstrated effects of various small changes of conditions on the molting sequence and developmental duration of laboratory-reared larvae, it is quite possible that their life-histories may be inapplicable to larvae in the field. The matter is further discussed by Rees (1959). In the case of *P. planipes* larvae, however, all of the stages, except Stage VI, seen in the laboratory, including evidence for a complex of Stage IV sub-stages, can be found in larvae from the plankton. The number and detailed morphology of the Stage IV sub-stages which larvae pass through in the ocean may well differ from the number passed through by larvae in laboratory experiments, and the number of sub-stages may even vary from one part of the ocean or one season of the year to another.

The irregularities in the number of molts in the larval phase shown by *P. planipes* may be found in other anomuran crabs. Sub-stages, however, are difficult to detect in morphological studies of larvae taken from plankton collections. Johnson and Lewis (1942) found a "lower Stage IV" in the larval stages of *Emerita analoga*, based on larvae from the plankton. This lower stage is best interpreted as a sub-stage and is an indication that sub-stages occur naturally in the field. Rees (1959) noted that larvae of *Emerita talpoida* reared in the laboratory may pass through either 6 or 7 molts before becoming megalops. Similar results were noted in laboratory-reared larvae of *E. analoga* by Dr. Ian Efford (unpublished results, personal communication). A. Provenzano (personal communication) has observed a varying number of molts in larvae of some pagurid crabs from Florida cultured by him. Costlow (personal communication), however, has observed variation in the number of larval molts in only two species of Brachyura out of a total of about 20 species—both Portunidae; variation occurred only occasionally and resulted in a form with reduced viability which only rarely developed to the megalops stage. Broad (1957a, 1957b) found that the number of larval molts varied in *Palaemonetes pugio*, a decapod macruran, depending on the type of food given the larvae.

Stage VI, which appeared in Experiment 1, has never been found in the plankton and appears to be the result of laboratory rearing conditions. It is possible that it occurs in nature under certain conditions. Its existence would certainly support Gurney's contention that laboratory conditions produce aberrant larval forms. Kurata (1960) observed what may be a similar phenomenon in the advanced stages of two lithodid crabs (*Paralithodes camtschatica* and *P. brevipes*) reared in the laboratory. These stages were intermediate between the usual last larval stage and the glaucothoe.

The temperatures in Experiment 4 (12° to 22° C.) were selected because these are approximately the surface temperatures at the northern and southern ends of the crab's distribution in the adult phase. The few data available (unpublished) indicate that the larval distribution is similar to the adult distribution, with the greatest concentrations occurring along the western coast of southern Baja California. In that area winter temperatures may commonly be as high as 20° at the surface. Larvae at that temperature in the laboratory had a higher mortality rate than did those at lower temperatures. Possibly, larvae in the latitude of southern Baja California do not live at the surface but rather below the surface at a more optimal temperature. The winter breeding season may be correlated with higher larval survival in the laboratory at colder temperatures.

Because problems encountered by the larvae of many polychaetes and the decapod crustaceans are similar, in that they must transform from a pelagic phase to a benthic phase, it is tempting to speculate that decapod crustacean larvae such as those of *P. planipes* may respond to environmental parameters in a way similar to that demonstrated by Wilson for various polychaete larvae (*cf.* Wilson, 1952). He has shown that polychaete larvae are influenced by the nature of certain substrates so that they end the pelagic phase and become benthic. The presence of other substrates tended to prolong the larval phase. Experiments similar to Wilson's have not yet been performed on larval decapods, however, presumably because of the difficulties inherent in rearing them.

The authors wish to acknowledge the help given by Mrs. Dorothy Walton and Mr. John Nordback, who acted as technical assistants during parts of the study. Dr. Maurice Blackburn provided financial support through Scripps Tuna Oceanography Research Program for some of the work, and his help is gratefully acknowledged.

SUMMARY

1. The young of *Pleuroncodes planipes* pass through a series of five morphologically discrete zoeal larval stages after hatching, and, except in Stage IV, the larvae change from one stage to the next by a single molt. Larvae in Stage IV may molt from four to eight times without greatly altering their basic morphology. There is evidence from laboratory culturing that the number of these sub-stages may be influenced by the temperature at which the larvae develop, with higher temperatures causing more sub-stages.

2. The duration of the larval phase is influenced by the temperature at which the larvae are reared, and the rate of development follows a Q_{10} of about 1.9. The larval duration is also influenced by rearing conditions other than temperature, for the size of the larval rearing container or the presence of other larvae in the container has also been shown to influence the duration of the larval phase.

3. A larval stage was seen in the laboratory which has not been found in nature, and it is probable that the stage was an artifact of laboratory culturing conditions.

4. It may be generalized that variation in the number of larval molts is widespread in the Anomura; variation does not commonly occur in brachyuran Crustacea.

LITERATURE CITED

Boyd, C. M., 1960. The larval stages of *Pleuroncodes planipes* Stimpson (Crustacea, Decapoda, Galatheidae). *Biol. Bull.,* **118**: 17–30.

Boyd, C. M., 1963. Growth rates, distribution and notes on the general biology of a marine decapod crustacean, *Pleuroncodes planipes* Stimpson (Galatheidae). *U. S. Fish and Wildlife Service Fishery Bulletin;* in press.

Broad, A. C., 1957a. Larval development of *Palaemonetes pugio* Holthuis. *Biol. Bull.,* **112**: 144–161.

Broad, A. C., 1957b. The relationship between diet and larval development of *Palaemonetes. Biol. Bull.,* **112**: 162–170.

Costlow, J. D., Jr., C. G. Bookhout and R. Monroe, 1960. The effect of salinity and temperature on larval development of *Sesarma cinereum* (Bosc) reared in the laboratory. *Biol. Bull.,* **118**: 183–202.

Coffin, H. G., 1958. The laboratory culture of *Pagurus samuelis* (Stimpson) (Crustacea, Decapoda). *Walla Walla College Publications, Dept. Biol. Sciences and Biol. Station,* No. **22**: 1–5.

Gurney, Robert, 1942. Larvae of decapod Crustacea. Ray Society, London. 306 pp.

Johnson, M. W., and W. M. Lewis, 1942. Pelagic larval stages of the sand crabs *Emerita analoga* (Stimpson), *Blepharipoda occidentalis* Randall, and *Lepidopa myops* Stimpson. *Biol. Bull.,* **83**: 67–87.

Kurata, H., 1960. Last stage zoea of *Paralithodes* with intermediate form between normal last stage zoea and glaucothoe. *Bull. Hokkaido Reg. Fish. Res. Lab.,* **22**: 49–56.

Rees, G. H., 1959. Larval development of the sand crab *Emerita talpoida* (Say) in the laboratory. *Biol. Bull.,* **117**: 356–370.

Wilson, D. P., 1952. The influence of the nature of the substratum on the metamorphosis of the larvae of marine animals, especially the larvae of *Ophelia bicornis* Savigny. *Ann. Inst. Oceanographique,* **27**: 49–156.

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HIGH TEMPERATURE TOLERANCE AND THYROID ACTIVITY IN THE TELEOST FISH, TANICHTHYS ALBONUBES[1]

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Upper lethal temperatures have been ascertained for many species of fish (Loeb and Wasteneys, 1912; Hathaway, 1927; Sumner and Doudoroff, 1938; Brett, 1941, 1944, 1946, 1952; Doudoroff, 1942; Fry *et al.,* 1942, 1946). Different species, when tested under comparable conditions, exhibit characteristic diverse high-temperature death points which are often clearly related to the conditions of life to which the species are adapted. However, the precise mechanism by which death is caused at high temperature in ectotherms is still not understood (Fisher, 1958; Precht, 1958). The experiments of Fortune (1955) led to the conclusion that increased activity of the thyroid gland with increasing environmental temperature may be an important factor in the thermal death of fishes. Fortune reported that specimens of *Phoxinus* (*phoxinus*) *laevis* kept in .05% thiourea solution at 23° C. for three days and then subjected to a 10° temperature rise over a two-day period were able to survive this treatment and to live indefinitely at 33° C. On the other hand, specimens given no thiourea treatment during the three-day period of acclimation at 23° C. all succumbed by the time the temperature reached 24° C. Inhibition of thyroid activity by thiourea treatment thus enabled *Phoxinus* to tolerate a temperature 9° C. above that which would otherwise have been lethal. Fortune also reported that the thermal range of *Lebistes reticulatus* could be similarly extended by thiourea treatment.

Earlier work by Evropeitzeva (1949) on thiourea-treated fry of *Coregonus* is in agreement with Fortune's findings but several other investigations have given diametrically opposed results. LaRoche and Leblond (1954) found that the parr of *Salmo salar* thyroidectomized with radioiodine had a lower tolerance for high temperature than did untreated controls. Auerbach (1957), working with *Lebistes reticulatus, Platys variatus,* and *Leuciscus rutilus,* and Theobald (1959) with *Gambusia affinis* found that thiourea treatment decreased the high-temperature tolerance in these species. Suhrmann (1955) reported that the goldfish, *Carassius vulgaris,* has an increased high-temperature tolerance if kept in thiourea at a low acclimation temperature but has a decreased tolerance if the acclimation temperature is high.

This paper reports the results of a study of the effects of thiourea treatment upon high-temperature tolerance and upon thyroid histology in the cyprinid, *Tanichthys albonubes.* Effects upon pituitary histology, which were also investigated, will be considered in a separate publication.

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Materials and Methods

Tanichthys albonubes Lin, the common whitecloud, is a small tropical oviparous fresh-water fish. A total of about 300 specimens was used in this study. The average length of the fish used, measured from the tip of the snout to the hypural plate, was 2.6 cm. ± 0.2 cm.

During the phases of the experiments described below, the control whiteclouds were kept in dechlorinated tap water and the experimental fish were kept in 0.05% thiourea contained in either 5-gallon aquaria or polyethylene pails. These media received continual aeration and were changed twice a week. The fish were fed daily with dry tropical fish food. Prior to the actual experiments several series of pilot experiments were carried out before adopting the subsequent methods regarding the concentration and period of treatment with thiourea, the acclimation temperature to be used, and the method of determining the upper lethal temperature.

1. *Pre-acclimation period*

The pre-acclimation period was designed to bring about hypofunction of the thyroids of the experimental fish. To accomplish this, no more than 75 experimental fish were placed in a 5-gallon aquarium containing 18 liters of 0.05% thiourea and kept at room temperature (21° C.). An equal number of controls were also kept at room temperature in an equal volume of water in a similar aquarium. This phase was continued for 30–40 days. At the end of this period, histological examination of the thyroids of a few treated animals revealed that thyroid hypofunction had been effected.

2. *Acclimation at low temperature*

After the pre-acclimation treatment, the control and experimental whiteclouds were kept in 11-quart polyethylene buckets containing thiourea solution or water. Each bucket contained no more than 40 fish. The buckets were placed in a 50-gallon Aminco Laboratory Bath (refrigerator type) regulated to maintain a temperature of 15.0° C. ± 0.05° C., and kept there from 40 to 60 days. During the pre-acclimation and acclimation phases, fish mortality was negligible.

3. *Upper lethal temperature determination*

A common method for determining the upper lethal temperature of fish is to directly transfer the test animals from the acclimation tank to the high-temperature tank and then to calculate the temperature at which half of the animals die in a given period of time. The periods of exposure to the high temperature vary widely in different studies. A number of investigators have used a 24-hour period (Black, 1953; Theobald, 1959). Following these authors, the lethal temperature in this study was considered to be that temperature at which 50% of the fish succumbed and 50% survived after 24 hours' exposure. High temperatures at which the whiteclouds were tested ranged from 29.5° C. to 31.5° C. The water baths used to test the fish at high temperatures were Aminco Laboratory Baths which could maintain water temperatures within 0.05° C.

It was observed in preliminary experiments that when whiteclouds were directly transferred from the acclimation tank to a high-temperature tank, symptoms of shock, such as rapid movement of the opercula, quick spiral swimming, and partial or total loss of equilibrium, occurred. To minimize this shock the method was modified as follows. Water baths were set at the high temperatures at which fish were to be tested. Samples of control and experimental fish (4–6 fish per sample) from the acclimation bath were placed in small polyethylene pails containing either water or 0.05% thiourea already cooled to 15.0° C. The pails were then transferred to a hot-water bath set at 45.0° C., and left there until the temperatures at which the fish were to be tested (29.5° C. to 31.5° C.) were reached. This required 15 ± 0.5 minutes. The pails were then placed in the appropriate water baths set at the temperature at which that particular group of fish was to be tested. The fish were left at this temperature for 24 hours. This substitution of a method of increasing the temperature over a period of 15 minutes for the direct transfer method originally used eliminated all signs of shock.

4. *Methods of fixation and staining*

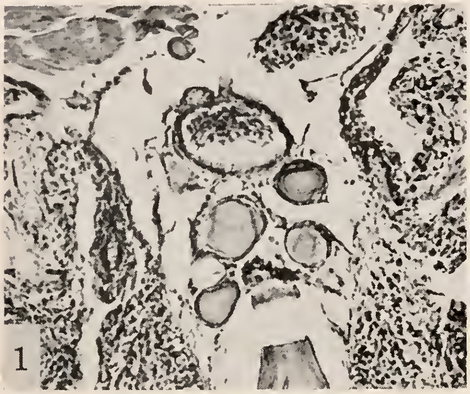
After 24 hours' exposure to the upper lethal temperature, surviving control and experimental fish and an identical number of specimens from corresponding solutions in the 15.0° C. acclimation bath were removed and anesthetized in 1/1000 tricaine-methane-sulfonate (MS-222). The MS-222 was warmed or cooled to the temperature from which the fish were taken. The lower jaws of these fish were fixed in Bouin's fluid and embedded in paraffin. Records were kept concerning the sex and maturity of all specimens. Serial sections of the thyroid glands from control and experimental fish from the same sex group and from all three phases of the experiment were stained simultaneously. The thyroids were stained in Gomori's chrome-alum-hematoxylin-phloxine.

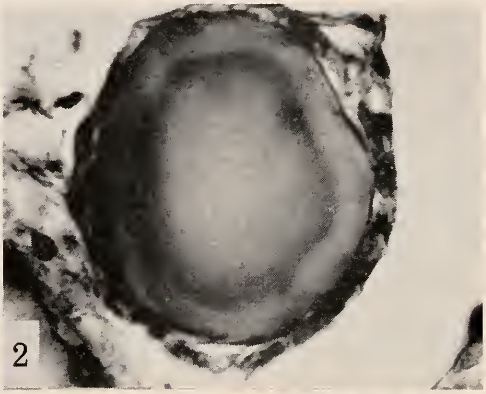
OBSERVATIONS

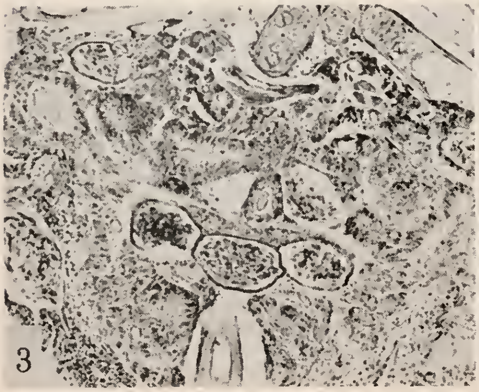
1. *Thyroid histology*

The thyroid histology of the experimental fish was significantly different from that of the control animals. The thyroids of control fish at the end of the various phases of the experiment showed no significant differences. Similarly, no significant differences were observed in the thyroid histology of the thiourea-treated fish after each of three experimental phases. The descriptions which follow are based on histological observations of the thyroids of control and experimental fish killed at the end of the acclimation phase of the experiment.

Examination of serial sections of individuals from the control groups showed that the thyroid of the whitecloud belongs to the non-encapsulated diffuse type found in most teleosts (Fig. 1). The follicles are scattered in the connective tissue beneath the floor of the pharynx but tend to accumulate around the ventral aorta and the aortic arches. These follicles are roughly spherical in shape. The average number of follicles found in a representative cross-section through the thyroid region of control fish is 9. Occasionally small capillaries are seen in contact with some of the follicles. The follicular epithelium is mostly squamous, although some of the small follicles have cuboidal to low columnar epithelium, the average height being 3.0 microns (Fig. 2). The squamous cells contain little cytoplasm, and their flattened, dark-staining nuclei occupy most of the cell. The cuboidal epithelial cells have round, usually basally located, nuclei and a small amount of slightly basophilic cytoplasm. No colloid droplets or vacuoles were observed in any of the follicular cells of the control thyroids. Except for a few of the small, more active follicles, the follicular lumina are large and filled with acidophilic, homogeneous, or granulated, non-vacuolated colloid.







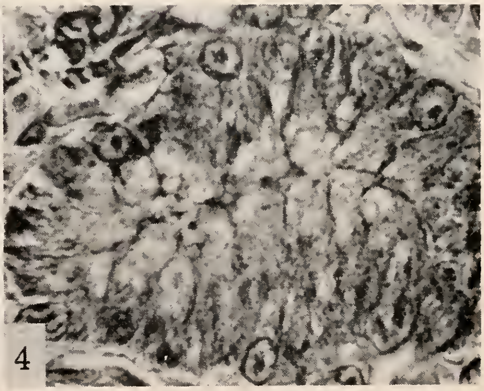


FIGURE 1. Section through thyroid region of an untreated control kept at room temperature (21° C.) for 30 days, then at 15° C. for an acclimation period of 60 days. 50 ×.

FIGURE 2. A single thyroid follicle from the specimen shown in Figure 1. 485 ×.

FIGURE 3. Section through thyroid region of a fish kept in 0.05% thiourea throughout a preacclimation period of 30 days at 21° C. and an acclimation period of 60 days at 15° C. 50 ×.

FIGURE 4. A single thyroid follicle from the specimen shown in Figure 3. 485 ×.

Examination of the thyroids of thiourea-treated fish reveals a histological picture quite different from that of the controls (Fig. 3). A great increase in the number and size of follicles has occurred so that the follicles are more compactly arranged and occupy a large portion of the sub-pharyngeal region. The average number of follicles found in a representative cross-section through the thyroid region of experimental fish is 25. The follicular epithelium is columnar and averages 19.5 microns in height. The cytoplasm of the thyroid epithelium contains large vacuoles and intracellular colloid droplets not found in controls (Fig. 4). Large, round, vesicular, basally located nuclei containing prominent nucleoli are found in most of the follicular cells. Many follicles are collapsed while those which are not contain only a small amount of basophilic, vacuolated colloid. A hyperemic state is evidenced by the increase in the number and size of interfollicular capillaries and other small blood vessels when compared with the controls.

TABLE I

Survival of fish previously acclimated at 15° C. and then exposed for 24 hours to the high temperatures indicated

Test		29.5°	30.0°	30.5°	31.0°	31.5°
Controls	#1			5/6	6/6	0/3
	#2				3/6	0/6
	#3			3/6	3/5	
	#4				3/6	
	#5				2/6	
	#6				3/6	
	#7				0/6	
	#8				6/6	
Totals				8/12 (67%)	26/47 (55%)	0/9
Thiourea-treated	#1	3/6	3/4	0/4	0/4	
	#2		4/6	2/6		
	#3		3/6	2/6		
	#4		3/6	1/5		
	#5		4/5	0/5		
	#6		3/6			
	#7		1/6			
	#8		3/6			
Totals		3/6 (50%)	24/45 (53%)	5/26 (19%)	0/4	

2. *Upper lethal temperature*

Based on the method described above, the upper lethal temperature for control *Tanichthys albonubes* was found to be 31.0° C., while that for the experimental fish was 30.0° C. The results of the experiment are summarized in the accompanying table. The upper half of the table contains the data obtained for control fish while the lower half contains the data for experimental fish. The figures in the uppermost part of the table indicate the various high temperatures to which the fish were exposed. The equations under each temperature show the number of fish per sample which survived at that particular temperature for 24 hours. For example, in test #1 of the controls, 5 out of 6 fish survived at 30.5° C., 6 out of 6 survived at 31.0° C., and none out of three survived at 31.5° C. when left at these temperatures for 24 hours. In test #2 of the controls, three out of 6 fish survived 24 hours' exposure at 31.0° C., while all of the 6 fish placed in 31.5° C. died within 24 hours. Since the early tests indicated that the upper lethal temperature for the whitecloud was 31.0° C., the majority of the tests were made at this temperature. Totals of these tests show that 8 out of 12 fish, or 67%, survived 24 hours at 30.5° C., 26 out of 47, or 55%, survived 31.0° C., while 24-hour exposure to 31.5° C. proved lethal for all fish kept at this temperature. Similar comparisons may be made for the experimental animals by examining the lower part of the table. A comparison of the results obtained for controls and experimentals indicates that the controls had a greater capability to withstand high temperature than did the fish treated with thiourea. It should be mentioned that there was no significant difference in the survival in males and females.

Discussion

Piscine thermal tolerance extremes depend on previous acclimation temperatures. This fact is evident from the numerous thermal studies done on fish since the time of Loeb and Wasteneys (1912). The rate at which acclimation occurs, however, seems to differ widely in different fish. Some authors (Doudoroff, 1942; Brett, 1944, 1946) have found that gain of heat tolerance is more rapid than loss in the process of acclimation. Tsukuda (1960), on the other hand, reported that changes in cold tolerance are almost parallel to changes in heat tolerance in the guppy, *Lebistes reticulatus,* with both processes being relatively slow. Using approximately the same temperature change intervals Doudoroff (1942) and Brett (1944) found for *Girella nigricans* and *Phimephales pomelas,* respectively, that a period of 20 days or more was required to completely acclimate these fish at low temperatures. About 35 days are required to acclimate male guppies at low temperatures (Tsukuda, 1960). Although no rate of acclimation was ascertained for the whitecloud in the present experiments, in view of the findings of Doudoroff, Brett and Tsukuda, and of the relative stability obtained in the lethal temperature for the whitecloud, the 40–60 days' exposure to 15.0° C. seems to have been a sufficient period to bring about a stable physiological condition at the low temperature.

Under the conditions of this experiment the high-temperature death point for controls of the cyprinid, *Tanichthys albonubes,* was found to be 31.0° C. Brett (1956) tabulated the reported lethal temperatures for a number of species of fish. Members of the Cyprinidae recorded by Brett have upper lethal temperatures ranging from 28.9° C. to 32.8° C. Hence, the upper lethal temperature ascertained for the whitecloud here is in agreement with the range of upper thermal limits recorded for other members of the family.

With a few exceptions, the thyroid of teleosts consists of unencapsulated follicles scattered individually or in small groups in the connective tissue along the ventral aorta and branchial arteries in the lower jaw of the fish. The thyroid morphology of *Tanichthys albonubes* is consistent with this general pattern. This diffuse nature of the teleost thyroid renders complete surgical extirpation of the gland impossible. To study the effect of hypothyroidism in these animals one must have recourse to chemical inhibitors or to thyroidectomy by radioiodine. Thiourea has been used on teleosts by a number of investigators (Scott, 1953; Fortune, 1953, 1955, 1956, 1958; Frieders, 1949, 1954; Suhrmann, 1955; Auerbach, 1957; Theobald, 1959; and others). Common effects of thiourea treatment on the thyroid histology of fish include increased vascularization in the thyroid area, hyperplasia and hypertrophy of the follicular epithelium, and a loss of stored colloid. The degree to which these results are achieved depends on the species of fish used, the concentration of thiourea, the length of the treatment and other factors. This hyperactive appearance of the thyroid after thiourea treatment may be explained on the basis of the thyroid-pituitary relationship which exists in fish as in other vertebrates. Thiourea prevents the thyroid from synthesizing its hormone so that, as soon as the supply of circulating hormone present at the beginning of thiourea treatment falls below a certain level, the pituitary begins to secrete thyrotrophin to activate the thyroid to produce more hormone. The thyroid, however, because of continued treatment, remains hypofunctional despite the fact that it is hyperactive. Nevertheless, in some species under certain conditions, prolonged treatment with antithyroid drugs may result in an "escape" from thyroid inhibition (Pickford and Atz, 1957). Frieders (1949) observed that the hyperplastic thyroid of *Trichogaster trichopterus* resulting from 0.0025% thiourea treatment at room temperature returned to a normal state after the fifth week of treatment. Similar reactions to thiourea were shown by Fortune (1958) to exist in *Phoxinus laevis.* The hyperemic, hyperplastic and hypertrophic condition of the thyroid following thiourea administration in the present work is clear evidence that the thyroid of the whitecloud was quite responsive to thiourea treatment. Since this hyperactive state persisted from the beginning of the acclimation at low temperature to the end of the experiment, it is concluded that there was no "escape" from the thyroid inhibition, and that the thyroid hormone was totally or to a great degree suppressed in the experimental animals during this time, a desired condition for the experiment.

The dramatic results obtained by Fortune (1953, 1955) served as a basis for the hypothesis that decreased thyroid function in teleosts at high temperature is a significant factor in causing death at the lethal temperature. Fortune (1955) kept *Phoxinus (phoxinus) laevis* in thiourea at 23° C. for three days and then subjected them to a rise in temperature to 33.0° C. over a period of two days. The experimental fish survived indefinitely at this increased temperature and appeared normal, while non-thiourea-treated fish died under such conditions between 23.0° C. and 24.0° C. Although no data were given, Fortune reported in the same paper that the thermal range of *Lebistes reticulatus* could also be extended by thiourea treatment.

Other experiments involving high-temperature tolerances and thyroid hypofunction in teleosts have produced conflicting results. Evropeitzeva (1949) reared six-day-old fry of *Coregonus lavaratus ludoga* for 17 days in thiourea at room temperature and then exposed 100 control and experimental animals to 29.0° C. for 5 minutes. Ninety-seven per cent of the control animals died while 97% of the experimental animals survived the 5-minute exposure. These results seem to agree with Fortune's hypothesis. The studies of LaRoche and Leblond (1954) and Auerbach (1957) on other young fish, however, oppose the work of Evropeitzeva. LaRoche and Leblond thyroidectomized young Atlantic salmon, *Salmo salar,* by radioiodine and found that thyroidectomy impaired the ability of these salmonid parr to withstand rising temperatures (5.0° C. to 10.0° C.), while the thyroidectomized fish receiving thyroid material in their diet survived such rising temperatures. Auerbach acclimated young *Xiphophorus helleri* at 15.0° C. and 25.0° C., and then put some of the fish in 0.15% thiourea at the same temperatures for 14 days. She tested the control and experimental fish at high temperatures and found that the cold- and warm-adapted controls evidenced heat coma at higher temperatures than did the cold- and warm-adapted thiourea-treated fish.

Using adult fresh-water fish, Suhrmann (1955) decreased thyroid function in *Carassius vulgaris* by keeping these fish in thiourea at 5.0° C. and 26.0° C., and then found that heat coma occurred in the cold- and warm-acclimated fish at 31.4° C. and 35.2° C., respectively. By comparing her results with the findings of Christophersen and Precht (1952) for untreated *Carassius vulgaris* kept at the same acclimation temperatures, she deduced that thiourea increased upper temperature tolerance for cold-acclimated fish, but decreased it for the warm-acclimated ones.

The work of Auerbach (1957) and Theobald (1959) on other adult fresh-water fish does not agree with Fortune's results. Auerbach reported that controls for *Lebistes reticulatus* and *Platy variatus* acclimated at 25.0° C. and 15.0° C. had higher heat coma temperatures than experimental animals acclimated at the same temperatures but subjected to a 14-day treatment of 0.15% thiourea. Theobald administered various concentrations of thiourea or thyroid-stimulating hormone (TSH) to *Gambusia affinis* for 5 weeks at 25.0° C., and then acclimated the fish to 30.0° for one week. He found that the upper lethal temperatures for control, thiourea-treated, and TSH-treated animals were 37.4° C., 35.6° C. and 37.9° C., respectively.

Auerbach (1957) reported that the marine fish *Leuciscus rutilus,* if treated with thiourea, could not stand as high temperatures as non-treated fish. She kept a group of this species at 5.0° C. for 2–4 weeks and a group at 20.0° C. for 1–2 weeks and then put half of each group in 0.15% thiourea for 14 days at these temperatures. She found that cold-adapted controls had a heat coma temperature range of 28.1° C. to 29.0° C., while the cold-adapted thiourea-treated fish had a heat coma temperature range of 26.3° C. to 27.0° C. The warm-adapted controls and thiourea-treated fish had heat coma ranges of 29.7° C. to 30.8° C. and 27.6° C. to 28.4° C., respectively.

The upper lethal temperature for whiteclouds exposed to 0.05% thiourea under the conditions of the present experiment was found to be 30.0° C., whereas the upper lethal temperature for untreated control fish was 31.0° C. Thus, these results are contrary to those of Fortune (1955) for *Phoxinus* and *Lebistes,* and indicate that thiourea treatment slightly decreased the ability of *Tanichthys albonubes* to withstand high lethal temperatures.

SUMMARY

1. The thyroid of *Tanichthys albonubes* was rendered hypofunctional by 30–40 days' immersion in 0.05% thiourea. This treatment affected the thyroid histology to a significant degree, resulting in hyperemia, follicular hyperplasia, and cellular hypertrophy with a loss of stored colloid.

2. After each group had been acclimated at 15.0° C., the upper lethal temperatures were determined for the thiourea-treated and control fish. The upper lethal temperature of the controls was 31.0° C. and that of the experimentals was 30.0° C. This indicates that in this species decreased thyroid function does not result in increased high-temperature tolerance but, in fact, slightly decreases it.

LITERATURE CITED

Auerbach, M., 1957. Hat die Schildrüse für die Temperaturadaptation der Fische eine Bedeutung? *Zeitschr. Fisch. Hilfswiss.*, 6: 605–620.

Black, E. C., 1953. Upper lethal temperatures of some British Columbia freshwater fishes. *J. Fish. Res. Bd., Canada,* **10**: 196–210.

Brett, J. R., 1941. Tempering versus acclimation in the planting of speckled trout. *Trans. Amer. Fish. Soc.,* **70**: 397–403.

Brett, J. R., 1944. Some lethal temperature relations of Algonquin Park fishes. *Pub. Ont. Fish. Res. Lab.,* **63**: 1–49.

Brett, J. R., 1946. Rate of gain of heat-tolerance in goldfish (*Carassius auratus*). *Pub. Ont. Fish. Res. Lab.,* **64**: 9–28.

Brett, J. R., 1952. Temperature tolerance in young Pacific salmon, genus *Oncorhynchus. J. Fish. Res. Bd., Canada,* **9**: 265–323.

Brett, J. R., 1956. Principles of thermal requirements of fish. *Quart. Rev. Biol.,* **31**: 75–87.

Christophersen, J., and H. Precht, 1952. Untersuchungen zum Problem der Hitzeresitenz. I. Versuche an Karauschen (*Carassius vulgaris* Nils.). *Biol. Zentralbl.,* **71**: 313–326.

Doudoroff, P., 1942. The resistance and acclimatization of marine fishes to temperature changes. I. Experiments with *Girella nigricans* (Ayres). *Biol. Bull.,* **83**: 219–244.

Evropeitzeva, N. V., 1949. Influence of thiourea on development of the thyroid gland in sigludoga (*Coregonus lavarctus ludoga* Pol.). *Dokl. Akad. Nauk., USSR.,* **68**: 977–980. (In Russian.)

Fisher, K. C., 1958. An approach to the organ and cellular physiology of adaptation to temperature in fish and small mammals. *In:* Prosser, C. L. (ed.); Physiological Adaptation. New York, Ronald Press.

Fortune, P. Y., 1953. Thyroid activity in teleosts. *Nature,* **171**: 483–484.

Fortune, P. Y., 1955. Comparative studies of the thyroid function in teleosts of tropical and temperate habitats. *J. Exp. Biol.,* **32**: 504–513.

Fortune, P. Y., 1956. An inactive thyroid gland in *Carassius auratus. Nature,* **178**: 98.

Fortune, P. Y., 1958. The effect of temperature changes on the thyroid-pituitary relationship in teleosts. *J. Exp. Biol.,* **35**: 824–831.

Frieders, F., 1949. The effect of thiourea and phenylthiourea on growth and pigmentation of several species of fish. Master's dissertation, The Catholic University of America.

Frieders, F., 1954. The effects of thyroid-inhibiting drugs on some tropical fish. *Catholic Univ. Amer. Biol. Stud.,* **31**: 1–37.

Fry, F. E. J., J. R. Brett and G. H. Clawson, 1942. Lethal limits of temperature for young goldfish. *Rev. Canadienne de Biol.,* **1**: 50–56.

Fry, F. E. J., J. S. Hart and K. F. Walker, 1946. Lethal temperature relations for a sample of young speckled trout, *Salvelinus fontinalis. Pub. Ont. Fish. Res. Lab.,* **66**: 1–35.

Hathaway, E. S., 1927. Quantitative study of the changes produced by acclimatization on the tolerance of high temperatures by fishes and amphibians. *Bull. U. S. Bur. Fish.,* **43**: 169–192.

LaRoche, G., and C. P. Leblond, 1954. Destruction of thyroid gland of Atlantic salmon (*Salmo salar* L.) by means of radioiodine. *Proc. Soc. Exp. Biol. Med.,* **87**: 273–276.

Loeb, J., and H. Wasteneys, 1912. On the adaptation of fish (*Fundulus*) to higher temperatures. *J. Exp. Zool.,* **12**: 543–557.

Pickford, G. E., and J. W. Atz, 1957. The Physiology of the Pituitary Gland of Fishes. New York Zoological Society, New York. 613 pp.

Precht, H., 1958. Concepts of the temperature adaptation of unchanging reaction systems of cold blooded vertebrates. *In:* Prosser, C. L. (ed.) ; Physiological Adaptation. New York, Ronald Press.

Scott, J. L., 1953. The effects of thiourea treatment upon the thyroid, pituitary and gonads of the zebra fish, *Brachydanio rerio. Zoologica,* **38**: 53–62.

Suhrmann, R., 1955. Weitere Versuche über die Temperaturadaptation der Karauschen (*Carassius vulgaris* Nils). *Biol. Zentrabl.,* **74**: 432–448.

Sumner, F. B., and P. Doudoroff, 1938. Some experiments on temperature acclimatization and respiratory metabolism in fishes. *Biol. Bull.,* **74**: 403–429.

Theobald, P. V. K., 1959. The relation of thyroid function to upper lethal temperature in *Gambusia affinis. Catholic Univ. Amer. Biol. Stud.,* **50**: 1–37.

Tsukuda, H., 1960. Temperature adaptation in fishes. IV. Change in the heat and cold tolerances of the guppy in the process of temperature acclimatization. *J. Inst. Polytechn. Osaka City Univ.,* **11**: 43–54.

PELAGIC LARVAE OF NERINIDES AGILIS (VERRILL)[1]

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While the authors were engaged in a study of polychaetous annelid reproduction and development, a larva similar to that of *Nerinides tridentata,* described by Hannerz (1956), began to appear in the plankton of the Mystic River estuary in June and continued to occur through August of 1962. *N. tridentata* has not been reported from the New England coast, but *N. agilis* is found in sandy beaches along the Atlantic seaboard (Verrill, 1873; Hartman, 1945; Carpenter, 1956). A three-day search of local beaches yielded but one young adult. Since ripe adults were not available for laboratory fertilization studies, development of this spionid polychaete has been described from specimens obtained from the plankton and reared in the laboratory through metamorphosis and until identification of the species was definite.

MATERIALS AND METHODS

Larvae were collected in a no. 10 plankton net in qualitative tows from the Mystic River estuary, Noank, Connecticut. Larvae believed to belong to the same species, but representing various stages of development, were separated from plankton samples, described, photographed and drawn. For examination, larvae were placed on microscope slides with small pieces of Saran Wrap used as cover slips (Dean and Hatfield, 1963). This was effective in quieting the larvae without apparent injury. Photographs showing the outline of an undistorted larva and revealing a certain amount of detail were obtained using a Polaroid camera with 10-second film mounted on a phase microscope. Additional detail was provided through further photographs, descriptions and quick drawings of characteristic parts. Composite outline drawings were made from the photographs and descriptions. Following examination some of the larvae were transferred to 5-cm. funnels (Wilson, 1952) used as rearing vessels, and raised to metamorphosis. Funnels contained filtered sea water and sediment of mixed particle sizes ranging from 53 to 590 μ. Funnels were immersed in a bath of running sea water, and the larvae were fed liver powder (Howie, 1958) once a week. Sea water in the funnels was changed daily.

The sea water used was filtered through a 47 μ Millipore filter and stored in a stoppered flask immersed in the same water bath as the funnels containing the larvae. Sediment was obtained from the Mystic River estuary, washed three times with distilled water, air-dried, sieved with standard screens to obtain the particle sizes desired and stored in stoppered jars.

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Observations and Discussion

Pelagic larvae of *N. agilis* should be easily distinguishable from all other polychaete larvae occurring in marine plankton of the New England region. The most apparent characteristic features of all planktonic stages are the acutely pointed prostomium and the typical opaque dark-brown to black gut. The prostomium has a protrusible finger-like process at its anterior extremity which is retracted by means of two longitudinal bands of muscles. The muscles arise on the anterolateral aspects of the mouth and insert at the anterior tip of the process. The latter is usually retracted when the larvae are preserved. In the late pelagic stages, *viz.,* 14- to 16-setiger larvae, the ability to retract this organ diminishes.

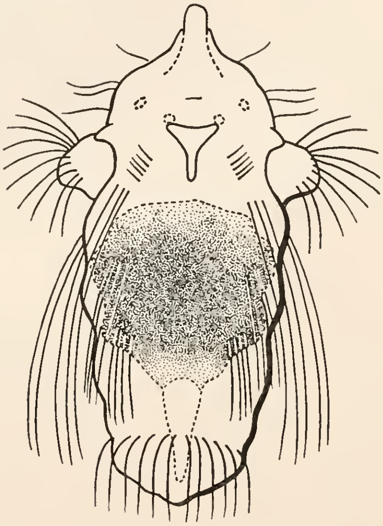

Figure 1. Ventral view of the 3-setiger larval stage of *Nerinides agilis*. Ciliation of the vestibule has been omitted.

Two lateral ear-like processes, called the umbrella by Häcker, as translated by Gravely (1909), are present from the 3- to 16-setiger stages and bear the prototrochal cilia. Four dark brownish-red eyes occur in almost a straight line and are present in all stages. Palp buds appear first in the 5-setiger stage and arise dorsolaterally from the umbrella. The palps grow slowly until about the 15- to 16-setiger stage, at which time their growth seems more rapid, a canal develops within them and their mobility increases.

Swimming setae occur in paired dorsal and ventral bundles on the first segment behind the umbrella, and are present up to the 16-setiger stage. At the 16- to 17-setiger stage swimming setae are lost, leaving shorter adult setae in their place. Setation on other segments consists of capillary notosetae on all segments and capillary neurosetae on setigers 1 through 11. On the twelfth setiger five hooded bidentate crotchets appear and continue on the segments posteriorly.

The mouth opens into the posterior part of the vestibule. The latter is an

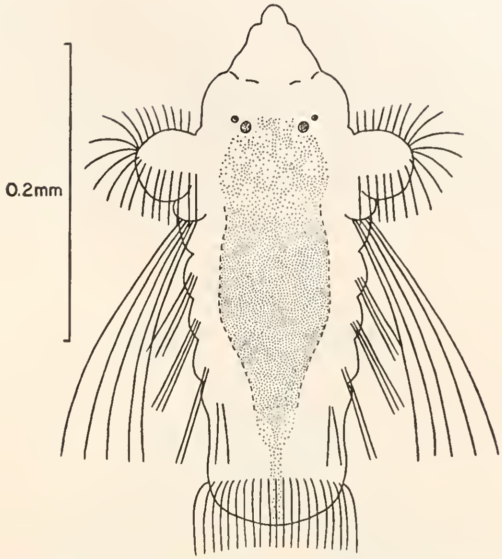

FIGURE 2. Dorsal view of the 5-setiger stage, showing palp buds posterior to the umbrella and the beginning of pharynx pigmentation. Sensory cilia have been omitted.

elongate depression on the antero-ventral aspect of the head region (Fig. 1) which is bordered posteriorly by the anterior part of the first setiger.

The pharynx appears as a large dark-brown to black pigmented area extending from the region of the peristomium posteriorly through setiger 2. The character-

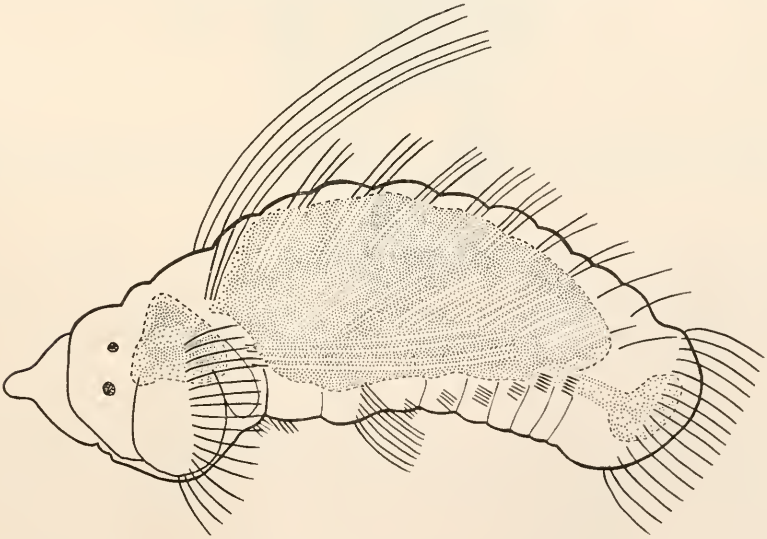

FIGURE. 3 Lateral view of the 10-setiger stage. The short cilia of the neurotroch can be seen on setigers 1 and 2. Gastrotrochs are present on setigers 3–9. Sensory cilia have been omitted.

istic reticulate pattern of the pigment of the pharynx begins to develop at the 5-setiger stage (Fig. 2). The density of pigment increases with larval development until at the 14-setiger stage it appears as dense as that of the stomach-intestine. Posteriorly from the pharynx an unpigmented oesophagus, about one segment in length, leads to the densely pigmented stomach-intestine. The latter region has lateral segmental diverticula (Fig. 4). The most posterior portion of the gut of young larvae is unpigmented (Fig. 1), while the gut in the region of the last few setigers of older larvae (Figs. 3, 4, 6) varies from partially to well pigmented. According to Hannerz (1956) the comparable region of *N. tridentata* is straight and unpigmented. In *N. agilis,* however, the gut is enlarged in the pygidial region and is pigmented (Figs. 3 and 4). In the present study the gut posterior to the last diverticula was ciliated.

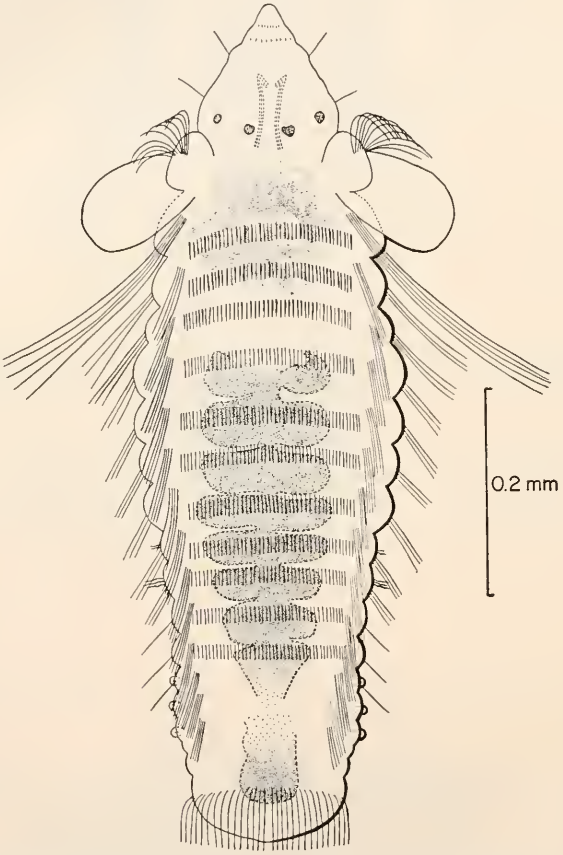

FIGURE 4. Fourteen-setiger stage in dorsal view. Nototrochs appear on setigers 1–11. Hooded crotchets appear in the neuropodia of the last three setigers.

Ciliation of *N. agilis* larvae is difficult to observe, due to the dense pigment of the gut. Cilia are of more than one type. Those of the prototroch, telotroch and the gastrotroch on setiger 3 are long and coarse. Cilia that appear on the prostomium are quite different, being variable in length, small in diameter and infrequent in movement. Wilson (1928) refers to this type as "sensory cilia." All other cilia in the larvae are short. Prototrochal cilia are discontinuous both dorsally and ventrally. The telotroch, however, consists of a completely encircling row of cilia. Gastrotrochs first appear in the 7-setiger larva and occur only on setigers 3 through 9, even in subsequent stages. There is one continuous band of cilia across the ventral surface of setiger 3. There are two patches per segment on setigers 4 through 9. The two patches are close together and near the ventral midline on setiger 4; on setigers 5 through 9 the patches become increasingly farther apart, until on setiger 9 they are lateral in position (Figs. 3 and 4).

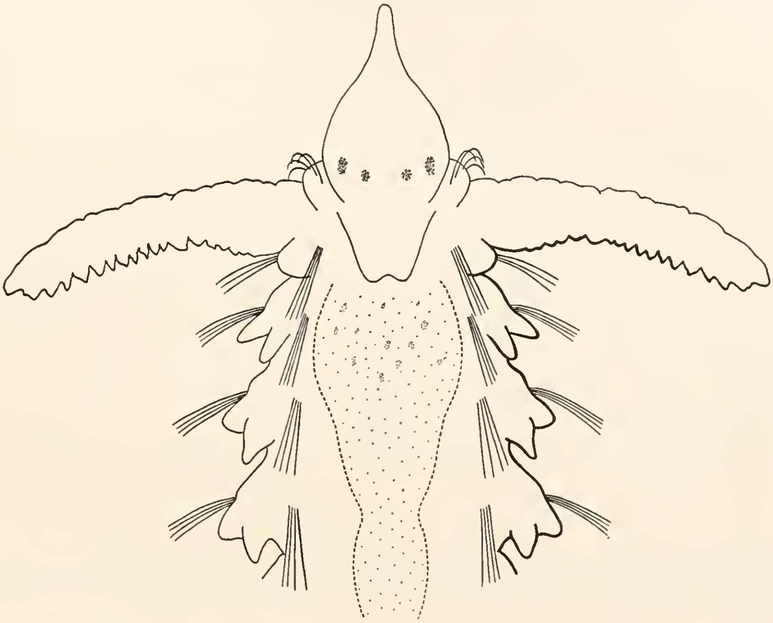

FIGURE 5. Anterior end of 17-setiger stage in dorsal view. The larva is ready to metamorphose. The larval setae of setiger 1 have been replaced by adult setae, and pharynx pigmentation is all but lost. Nototrochs and sensory cilia have been omitted.

The neurotroch (Fig. 3) makes its appearance between the 3- to 5-setiger stage. It extends from the well ciliated vestibule to setiger 2 and there ends in an inconspicuous ciliated pit.

Nototrochs were not observed in stages earlier than 14 setigers. At this stage cilia seemed to extend across the dorsal surface of segments 1 through 11 in a continuous band (Fig. 3). On 16-setiger stages examined for ciliation, nototrochs were not seen on setiger 1 but on setigers 2 through 16. In addition, these later stages showed the nototrochs to occur in three patches instead of one continuous band.

Several morphological changes take place in the larvae prior to metamorphosis.

The reticulate pigment pattern of the pharynx that is so characteristic of this larva begins to break down in the 16-setiger stage, and by the 17-setiger stage (Fig. 5) only a few dark spots smaller than the eyes remain. Adult setae are formed and accompany the swimming setae in the 16-setiger stage. The telotroch disappears and septa between the gut and body walls appear. Parapodia become more pronounced and buds of the dorsal and ventral postsetal lamellae appear. The dark pigmentation of the stomach-intestine begins to disappear progressively from the anterior toward the posterior region. Also, as the time of metamorphosis nears, the larval swimming setae are less firmly attached.

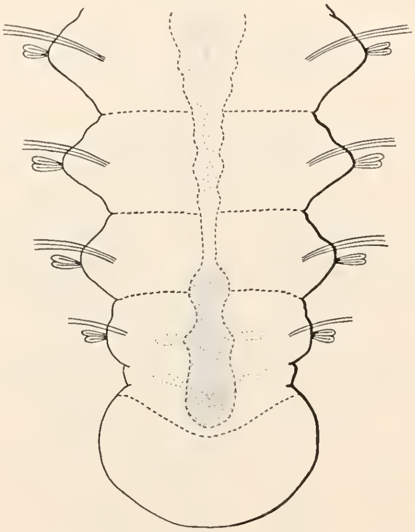

FIGURE 6. Posterior end in dorsal view of the same 17-setiger larva. Dense pigmentation of the posterior gut remains but the telotroch has been lost. Nototrochs have been omitted.

Metamorphosis occurs at the 17-setiger stage. Larvae were collected from the plankton up to but not exceeding the 17-setiger stage. The most advanced 17-setiger larvae had lost their telotrochs and larval setae, while their prototrochs, although still bearing functional cilia, were reduced to small lobes dorsal to the palpal bases (Fig. 5). All larvae reared successfully in the laboratory to benthic stages metamorphosed at the 17-setiger stage. Young benthic stages were reared to 19-setiger stages, at which time branchiae were formed on the second setiger.

Larvae reared in the laboratory were identical to larvae freshly obtained from plankton samples when specimens having the same number of segments were compared. In fact, the only variation noted during the study was a very slight difference in density of gut pigment between individuals. This was true of both reared specimens and those from the plankton.

The combination of the acutely pointed prostomium, the number and arrangement of eyes, the presence of branchiae beginning on setiger 2 and the bidentate hooded crotchets in the neuropodial segments show beyond any doubt that the larval development described is that of *Nerinides agilis*.

SUMMARY

Larvae of a spionid polychaete, occurring in the plankton of the Mystic River estuary from June through August, were isolated from plankton samples, photographed and drawn. Some of the isolated larvae were reared in the laboratory through metamorphosis and until the species was positively identified as *Nerinides agilis* (Verrill). Developmental stages of reared specimens agreed well with 3- to 17-setiger stages obtained from the plankton. Metamorphosis occurs at the 17-setiger stage. The 3-, 5-, 10-, 14- and 17-setiger stages are figured and pertinent larval characteristics described. The protrusible, acutely pointed prostomium, together with the deeply pigmented gut, are diagnostic features of all planktonic stages of this species.

LITERATURE CITED

CARPENTER, D. G., 1956. Distribution of polychaete annelids in the Alligator Harbor area, Franklin County, Florida. *Florida State Univ. Studies, No.* **22**: 89–110.

DEAN, D., AND P. A. HATFIELD, 1963. A method for holding small aquatic invertebrates for observation. *Turtox News,* **41**: 43.

GRAVELY, F. H., 1909. Studies on polychaete larvae. *Quart. J. Micr. Sci.,* **53**: 597–627.

HANNERZ, L., 1956. Larval development of the polychaete families Spionidae Sars, Disomidae Mesnil, and Poecilochaetidae N. Fam. in the Gullmar Fjord (Sweden). *Zool. Bidr. Uppsala,* **31**: 1–204.

HARTMAN, O., 1945. The marine annelids of North Carolina. *Duke Univ. Mar. Sta., Bull. No.* **2**: 1–54.

HOWIE, D. I. D., 1958. Dried organic substances as food for larval annelids. *Nature,* **181**: 1486–1487.

VERRILL, A. E., 1873. Report upon the invertebrate animals of Vineyard Sound and the adjacent waters, with an account of the physical characters of the region. *Rep. U. S. Fish. Comm. for 1871–72:* 295–778.

WILSON, D. P., 1928. The larvae of *Polydora ciliata* Johnston and *Polydora hoplura* Claparède. *J. Mar. Biol. Assoc.,* **15**: 567–603.

WILSON, D. P., 1952. The influence of the nature of the substratum on the metamorphosis of the larvae of marine animals, especially the larvae of *Ophelia bicornis* Savigny. *Ann. Inst. Ocean. Monaco,* **27**: 50–160.

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THE RELATION OF WHOLE-BODY I^{131} UPTAKE TO THYROID ACTIVITY IN THE DEVELOPING DOGFISH, SCYLIORHINUS CANICULA (L.)

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Inhibition of thyroid activity by various types of goitrogens is commonly ascertained by the presence of histological changes in the gland, such as hyperplasia, hypertrophy, and vacuolization of colloid, which are attributed to increased secretion of thyrotrophic hormone (TSH). Nevertheless, there are species among amphibians, *e.g., Diemyctylus viridescens* (Adams, 1946; Dent and Lynn, 1958; Lynn and Dent, 1961), and elasmobranchs, *e.g., Scyliorhinus canicula* (Tinacci, 1947; Olivereau, 1952; Dent and Dodd, 1961) and *Squalus suckleyi* (Pritchard and Gorbman, 1960), which have apparently a very low secretion rate of TSH or a very slow response of the thyroid to the pituitary hormone, for histological effects from administration of antithyroid substances are minimal. Studies with a radioactive isotope of iodine, I^{131}, indicate, however, that the common goitrogens are just as effective in inhibiting organic binding of iodine in these species as in those in which marked histological changes are produced (Olivereau, 1952; Dent and Lynn, 1958; Pritchard and Gorbman, 1960), and that TSH administration stimulates iodine-binding and, in high concentration and after prolonged treatment, enlargement of thyroid follicles also (Dent and Dodd, 1961).

Class experiments with tadpoles of *Rana pipiens* which had been immersed in 0.01%, 0.03%, or 0.05% thiourea solution for two weeks had revealed a higher whole-body I^{131} uptake than occurred in the controls. This phenomenon was ascribed to the presumed hyperplastic condition of the thyroid gland in the experimentals, although no histological examination was made (Bileau, 1956). Questions arose as to whether one could correlate whole-body iodine uptake with thyroid activity in animals in which goitrogens bring about thyroid inhibition only, without producing goiter, and also whether one could distinguish between different types of thyroid inhibitors by such means. The goitrogens commonly employed interfere with two mechanisms, *viz.,* the concentration of iodine (thiocyanate, perchlorate) and its organic binding (thiourea and its derivatives) (Pitt-Rivers and Tata, 1959).

Another aspect of thyroid activity which is of current interest is its relation to temperature in poikilotherms. Reports on teleost thyroid activity at both high and low temperatures are conflicting (Leloup and Fontaine, 1960; Olivereau, 1960). Possibly the only report on elasmobranch response is that of Dent and Dodd (1961), who found that for hatchlings of *Scyliorhinus canicula,* the amount of iodine bound by the thyroid is greater at a higher temperature.

The present study is concerned with the relation of whole-body I^{131} uptake to organic binding of I^{131} by the thyroid and to the histological condition of the gland, upon treatment with thiourea or perchlorate at different temperatures. The form chosen was the embryo of the spotted dogfish, *Scyliorhinus canicula* (L.). The thyroid in this species, which lacks a larval stage, in common with that of amniotes active at birth or hatching (Dimond, 1954; Mitskevitch, 1957), shows signs of activity comparatively early in development (Vivien and Rechenmann, 1954).

MATERIALS AND METHODS

Twenty-five dogfish embryos, all in the latter half of development, were obtained in July and August, 1960, from an outdoor tank at The Laboratory, Plymouth, England. The mature egg cases had been removed surgically from captured females, and the embryos were developing in running sea water.

For this work the specimens were removed from the egg cases and kept, each one separately, in fingerbowls with approximately 100 ml. sea water (salinity 33.3‰) which had been taken at a depth and was considered equivalent to filtered water. Thiourea or potassium perchlorate was added to the sea water of the experimentals to make a concentration of 0.05%. Series I was kept at room temperature, which varied from 18° to 23.5° C. In Series II and III, however, both controls and experimentals were divided into two temperature groups, one at room temperature and the other at 8° C. Of the six initial specimens of Series I, only two were alive after 11 days in the same medium, probably because of heavy bacterial growth in the water. A third living specimen (1C), taken from the aquarium, was removed from its egg case and added to the series just at the time of iodine administration. For Series II (10 specimens) and III (8 specimens), the medium, including the plain sea water of the controls, was renewed once a week for the fish kept in the cold and twice a week for those at room temperature. Only one fish in each of these last series died, both of them from the cold group.

Several of the larger fish were measured at the beginning of the experiment. It was difficult to keep them extended, however. Since I did not wish to run the risk of injuring them by removing them from the water, most of the fish were left unmeasured. Final measurements were made on the preserved specimens.

After 11, 13, or 21 days of goitrogen treatment, the specimens were exposed to a solution of NaI^{131} in carrier-free $Na_2S_2O_3$ diluted in plain sea water or drug solution to give an activity of 3 μc./ml. for Series I, and 1.8–1.85 μc./ml. for Series II and III. After 39 to 50 hours the fish were washed and placed in fresh goitrogen solutions or sea water. Then 24 hours later, after another washing, each fish was put into a 75- or 100-ml. beaker of sea water in a gamma Geiger well counter and a count of whole-body radiation made, 10 minutes for Series I, and three one-minute periods for Series II and III. The gamma Geiger detector consisted of a glass well about one-fourth inch thick, surrounded by five Geiger-Muller tubes, with the whole apparatus encased in heavy lead shielding. Its resolution time limited accuracy of counting to about 50,000 counts per minute. Background averaged 243 counts per minute.

In Series I, after the whole-body counts had been made, the yolk sac was tied off and severed from the body and counts made of the fish without yolk and of the yolk alone.

Immediately after the counting, the embryos of Series I and II were killed by immersion in Bouin's solution. Those of Series III were replaced in the same solutions they had been in since the cessation of the I^{131} treatment, and were counted again the next day. The specimens at 8° C. were then killed, whereas those at room temperature were held for three days longer at which time they were counted and preserved. The thyroid glands were then dissected out, embedded in polyester or ester wax, sectioned at 4–6 μ, and mounted serially on slides with 0.1% amylopectin solution (Steedman, 1957). Alternate slides were stained, the greater number of them with Gomori's chrome alum hematoxylin phloxin, and mounted, and the others were covered by Kodak Autoradiographic Stripping Film AR.10. After one to eight days the film-covered slides were developed and allowed to dry at room temperature. Later they were either stained with gallocyanin and metanil yellow (Bowie and Edmonson, 1960), and dried, then mounted with Xam and covered, or were mounted and covered without staining.

TABLE I

Series I. Whole-body uptake and thyroid binding of I^{131} in developing Scyliorhinus canicula, *after immersion in 0.05% thiourea solution for 11 days prior to exposure to 3 μc./ml. I^{131} for 43 hours and washing for 24 hours at room temperature (18–23.5° C.)*

Protocol number	Total length in mm.	Thyroid autoradiography*	Counts per minute, corrected for background		
			Entire fish	Body alone	Yolk sac alone
C4	86	++	53,663	53,391	4,798
1C	53	++	10,267	6,818	4,135
T4	80	–	5,633	5,671	643

* Two plus signs indicate a dense autoradiogram with stripping film; one plus sign, a faint autoradiogram; and a minus sign, no autoradiogram.

RESULTS

Tables I, II, and III summarize the greater part of the pertinent information on the effects of the antithyroid drugs and temperature. The whole-body I^{131} count cannot bear statistical analysis because of the different stages of development and probably also of thyroid activity, the small number of specimens in any one group, and the varying lengths of time between treatment and fixation. Also, since the autoradiographic film was exposed for different periods of time, no quantitative comparison of the amount of blackening over the thyroid gland is possible. Nevertheless, some patterns emerge which seem to deserve comment.

First of all, though, explanations of certain individual points are needed for clarification. The fish had a large yolk sac connected to the body by a cord (Figs. 1, 2) except for specimen 3P (Figs. 3, 4), which would probably have made its way out of the egg case about the time it was exposed to I^{131} (Ford, 1921). Five specimens (1C, 13P, 18C, 19P, and 20T) had still functional external gills at the termination of the study (Fig. 1), whereas for eight others (9C, 10P, 11T, 12C, 14T, 15C, 16P, and 17T) the gills atrophied during the experiment before the time of I^{131} administration (Fig. 2). The other individuals were

TABLE II

Series II. Whole-body uptake and thyroid binding of I^{131} in developing Scyliorhinus canicula, *after immersion in 0.05% thiourea solution for 13 days prior to exposure to 1.8 μc./ml. I^{131} for 39 hours and washing for 24 hours at either room temperature (18–23.5° C.) or 8° C.*

Protocol number	Total length in mm.		Temperature	Goitrogen treatment	Thyroid autoradiography*	Counts per minute, corrected for background
	Initial	Final				
7C	70	85	Rm.	none	++	40,504
5P**	70	87	Rm.	perchlorate		5,272
6T**	70	88	Rm.	thiourea		11,358
9C	50	63	8° C.	none	+	1,390
8P	65–70	70	8° C.	perchlorate	–	1,715
10P***	50	63	8° C.	perchlorate		
19P†		43	8° C.	perchlorate	–	514
4T	75	75	8° C.	thiourea	–	1,479
14T		54	8° C.	thiourea	–	846
20T		49	8° C.	thiourea	–	603

* See Table I.
** Gland lost in processing.
*** Died between 10 and 13 days.
† Exposed to only 0.9 μc./ml. I^{131}.

further developed at the beginning of the study. The thyroid gland, nevertheless, in even the smallest and presumably the least advanced individuals, had very definite follicular structure with colloid more or less vacuolated, and cuboidal cells varying in height even in a given gland. There are no striking histological differences between the controls and experimentals at 8° C. or between those of Series I and II (14 and 16 days of exposure to the goitrogens, respectively) at

TABLE III

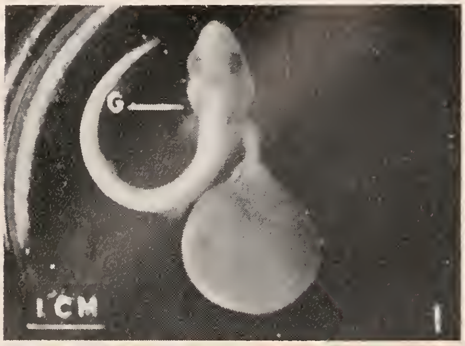
Series III. Whole-body uptake and thyroid binding of I^{131} in developing Scyliorhinus canicula, *after immersion in 0.05% thiourea solution for 21 days prior to exposure to 1.85 μc./ml. I^{131} for 50 hours and washing for 24 hours*

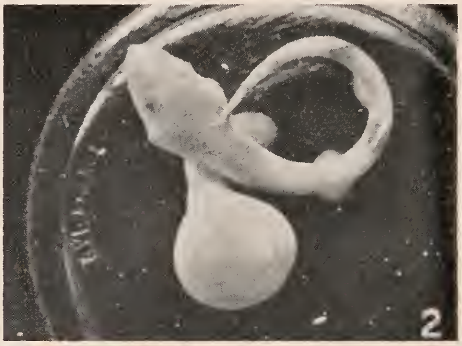
Protocol number	Total length in mm.		Temperature	Goitrogen treatment	Thyroid autoradiography*	Counts per minute, corrected for background and decay		
	Initial	Final				Initial	21.5 hrs. later	93 hrs. later
15C		80	Rm.	none	++	20,374	16,929	4,642
3P	85	98	Rm.	perchlorate	–	8,016	5,841	2,833
16P		85	Rm.	perchlorate	–	1,987	1,633	1,223
17T		76	Rm.	thiourea	–	5,488	3,725	1,375
12C**		66	8° C.	none				
18C		54	8° C.	none	+	1,084	761	
13P		60	8° C.	perchlorate	–	1,282	1,016	
11T		66	8° C.	thiourea	–	1,272	930	

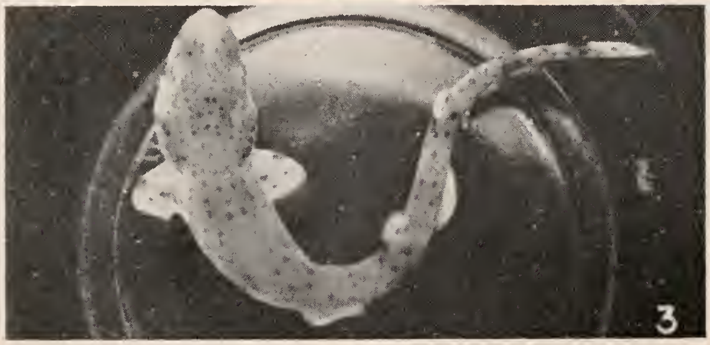
* See Table I.
** Moribund and preserved at 21 days.

room temperature. Nevertheless, somewhat higher cells, larger follicles, and more mitotic figures appear in the experimentals of Series III kept at room temperature (Figs. 5, 6, 7).

Growth of the developing fish was generally slowed down in the cold (Tables II and III), but not affected to any appreciable extent by either of the goitrogens. Exceptions to this, however, were specimens 9C and 10P, both of which increased in total length from 50 to 63 mm. during the cold-treatment. Activity was also diminished at a low temperature, the fish remaining very quiet as compared to those at room temperature, but there was no evidence of goitrogen influence on behavior.







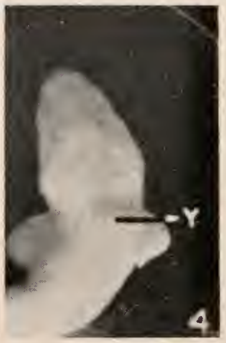


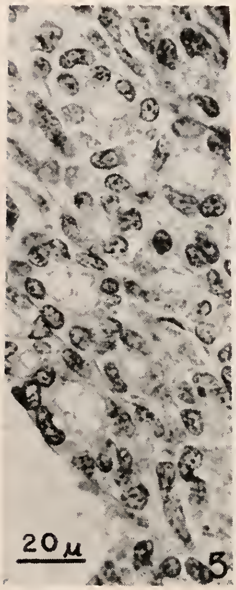
FIGURE 1. Specimen 18C, 54 mm., kept at 8° C. for 21 days, at the time of exposure to I^{131}. Notice the large yolk sac and the functional external gills (G).

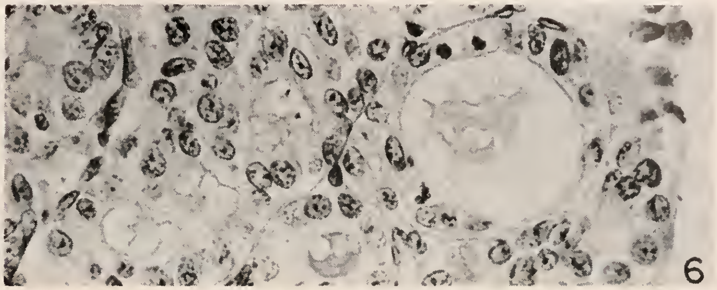
FIGURE 2. Specimen 15C, 80 mm., kept at room temperature. Notice a small yolk sac, the absence of external gills and the beginning of pigmentation.

FIGURE 3. Specimen 3P, 98 mm., kept in 0.05% $KClO_4$ at room temperature. It is probably a hatchling.

FIGURE 4. Ventral view of anterior region of specimen 3P. Notice the very small remnant of the yolk sac (Y).

In view of the size variations in the specimens, the I^{131} whole-body counts are probably not significantly different for the control and experimental animals kept at 8° C. In the case of those held at room temperature, however, an obvious pattern is indicated, for in the three sets (omitting specimen 3P, which was at least 10 mm. longer than the others and probably a hatchling at the time), the relation of I^{131} uptake was control > thiourea > perchlorate. The thyroid gland in the goitrogen-treated fishes, although it may have concentrated iodine, did not bind it, for there was no blackening of the autoradiographic film. In the controls at room temperature, however, the colloid of the gland appears well stocked with organic iodine (Figs. 8, 9), and even in those at 8° C., there is a sparse granulation of the autoradiographic film, indicating a low degree of iodine-binding (Figs. 10, 11).





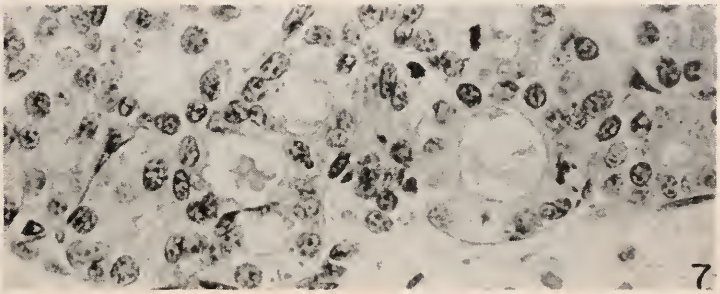


Figure 5. Section of thyroid gland of specimen 15C, 80 mm., kept at room temperature for 28 days. Cut at 6 μ. Stained with chrome alum hematoxylin phloxin. Compare with Figure 2.

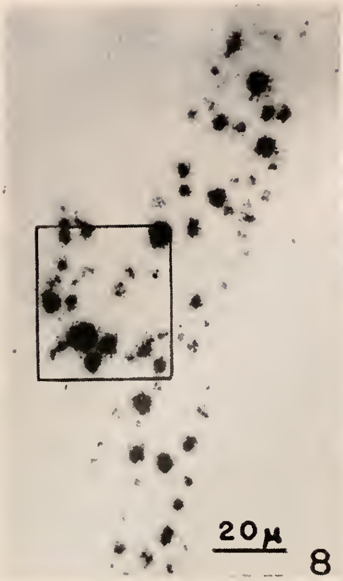
Figure 6. Section of thyroid gland of specimen 16P, 85 mm., kept in 0.05% $KClO_4$ at room temperature for 28 days. Staining and sectioning as in Figure 5. Notice the enlarged follicles, higher cells, and mitotic figures.

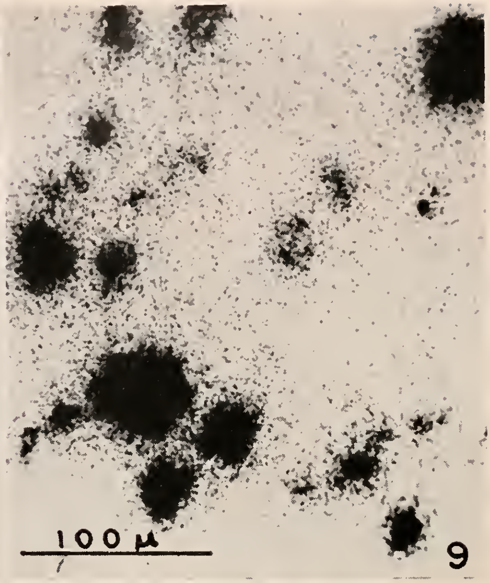
Figure 7. Section of thyroid gland of specimen 17T, 76 mm., kept in 0.05% thiourea at room temperature for 28 days. Notice the same characteristics as in Figure 6.

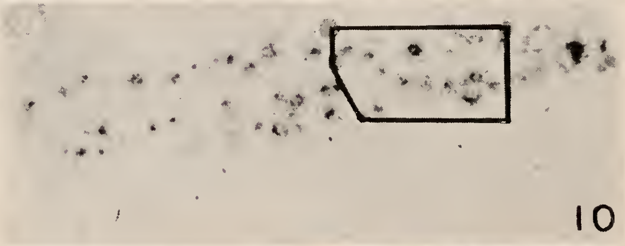
Finally, there is a marked difference in I^{131} excretion between the control and goitrogen-treated fish, as seen in Figure 12. Iodine removal from the control occurred at an arithmetic rate, whereas for the fishes exposed to perchlorate and thiourea the curve approached an exponential pattern.

Discussion

Iodine-131 in a concentration of 1.0 μc./ml. for 24 hours has been found suitable for absorption by fresh-water forms (Lynn and Dent, 1957). Since sea water contains so much iodine (50 μg./kg.), it was considered advisable to increase the amount of the radioactive form, and also the exposure time, in order to insure a measurable uptake. Actually, 1.0 μc./ml. would probably have been







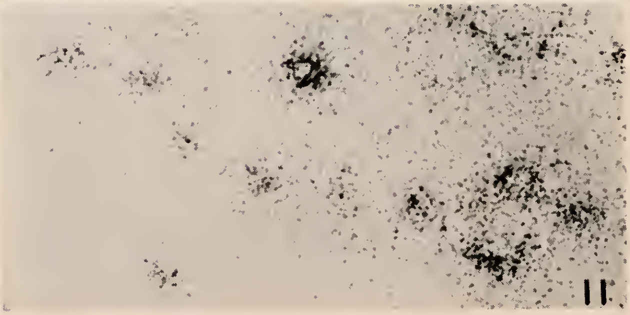


FIGURE 8. Autoradiogram from longitudinally sectioned thyroid of specimen 15C, 80 mm., kept at room temperature for 28 days. Compare with Figures 2 and 5. The animal was washed for 5 days after 50 hours' exposure to 1.85 µc./ml. I^{131} and then fixed. Four days later stripping film was applied to the sections and, after two days of exposure, developed. Cut at 6 µ.

FIGURE 9. Enlargement of a portion of Figure 8.

FIGURE 10. Autoradiogram from longitudinally sectioned thyroid of specimen 18C, 54 mm., kept at 8° C. for 25 days. The animal was washed for two days between I^{131} treatment and fixation. Four days later stripping film was applied to the sections and developed the next day. Compare with Figure 1. Same magnification and sectioning as Figure 8.

FIGURE 11. Enlargement of a portion of Figure 10. Same magnification as Figure 9.

satisfactory, for specimen 19P, not only the smallest of the fish, but also one treated with perchlorate, had a count almost three times background after exposure to only 0.9 μc./ml. The concentration of both thiourea and perchlorate (0.05%) was apparently optimal for *Scyliorhinus canicula,* for there was no evidence of toxicity effects, and the thyroid uptake of I^{131} seemed to be completely inhibited.

In *Scyliorhinus canicula,* thyroid activity begins very early in development. Vivien and Rechenmann (1954) report that at 25 mm., when the thyroid is still in the cord stage, it already binds iodine, but its role in embryonic life of elasmobranchs remains undetermined. Among the multiple effects of thyroid hormone during development are both gross changes, such as yolk sac retraction in teleosts and sauropsids (Dales and Hoar, 1954; Dimond, 1954), and histological changes, such as degeneration of the pronephros, as observed in amphibians (Lynn and Peadon, 1955).

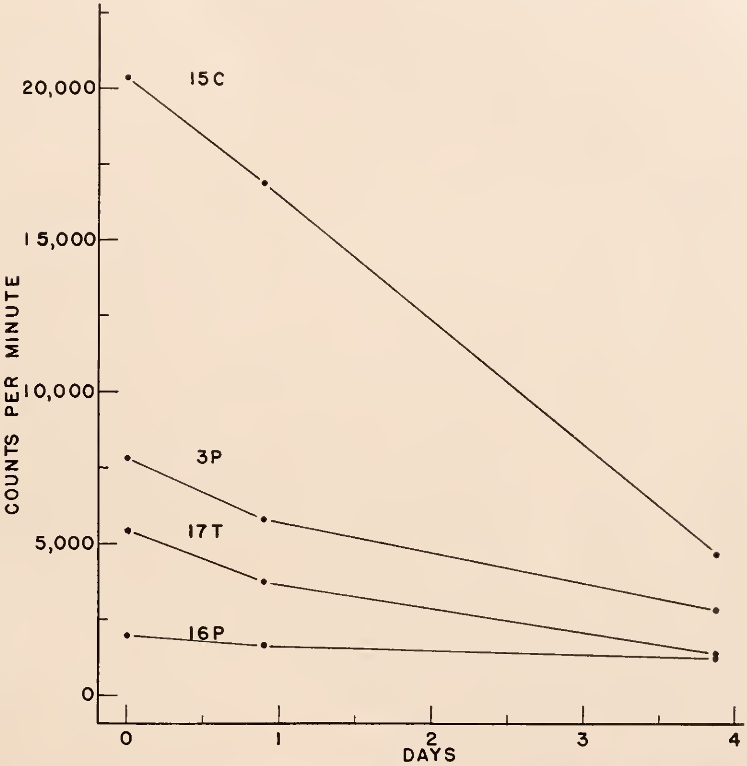


FIGURE 12. Elimination of I^{131} from four embryos of *Scyliorhinus canicula* which had been kept at room temperature for 21 days prior to exposure to 1.85 μc./ml. I^{131} for 50 hours and washing for 24 hours (Series III), as seen one and four days after the initial whole-body count. Counts per minute are corrected for background and decay.

In the present investigation, there were no obvious external differences between the controls and experimentals at a given temperature. Since only one specimen "hatched," no comparison can be made as to its relative speed of yolk-sac retraction, and the histological examination was limited to the thyroid. It seems reasonable to presume, however, that some morphological and physiological differences could be found by detailed study, since the thyroid gland is obviously very active during normal development.

Two facts concerning thyroid activity in the developing spotted dogfish at a low temperature (8° C.) stand out, *viz.,* that the gland concentrates and binds iodine to a degree sufficient for autoradiographic determination, and that thyroid hormone formation is so low as to evade detection in a whole-body count (Tables II, III; Figs. 10, 11). Whether thyroid action here is limited to a low rate of hormone production and storage, or whether release and distribution to the rest of the body occur as well, is undetermined. Dent and Dodd (1961) report a four-fold increase in tail muscle iodine of hatchlings with a change of temperature from 8.6° C. to 13.6° C.

Thiourea seems to have its typical action, *viz.,* prevention of organic binding of iodine, with little or no effect on iodine concentration, for although there was no reaction on the stripping film, yet the whole-body count in the case of the fish at room temperature was markedly higher than that of the perchlorate-treated specimens. An attempt was made to determine the relative amounts of thyroidal iodide by freeze-drying the glands, but an accident to the vacuum pump prevented completion of the processing.

The rate of thyroid hormone formation by dogfish embryos, and thus the histological response of the gland to thyroid inhibitors, appears to be directly related to the ambient temperature as well as to the state of development. Specimen 1C at room temperature, for example, had a whole-body count ten times greater than that of specimens 9C and 18C at 8°. Of course, it had been exposed to a somewhat higher concentration of I^{131}, but it is doubtful that that alone could account for the great difference.

Further, although both thiourea and potassium perchlorate prevented iodine binding, yet in only Series III at room temperature, exposed to the drugs for a total of 28 days, were the typical histological responses of hypertrophy and hyperplasia visible (Figs. 6, 7). Sixteen days' exposure (Series II) was not sufficient. Apparently even an active thyroid is slow as compared to that of homoiotherms.

Pritchard and Gorbman (1960) found about a 25% increase in cell height and "vacuolization" of colloid, as well as low I^{131} uptake in thyroid glands of near-term pups of *Squalus suckleyi*, after repeated injections with propylthiouracil. Tinacci (1947) observed some thyroid hyperplasia as well as hyperemia in the thyroid of *Mustelus laevis* after 25 days of oral administration of three different goitrogens, including thiouracil. On the other hand, Olivereau (1952), who administered thiourea or thiouracil to adults of *Scyliorhinus canicula* at 20° C., reports no histological differences between the controls and experimentals, other than the presence of almost pycnotic nuclei in the thyroid epithelium after 44 injections of thiouracil.

The pituitary hormone, however, is present and active in *Scyliorhinus canicula,* for hypophysectomy of developing pups prevents I^{131} fixation (Vivien and Rechenmann, 1954), and injection of mammalian TSH increases its organic binding in both hatchlings (Dent and Dodd, 1961) and adults (Leloup and Fontaine, 1960). Dent and Dodd have further shown that injection of ventral lobe extract from adult dogfish pituitaries over a period of three weeks results in an enhanced I^{131} binding, although the histological picture is not changed. Nevertheless, after three weeks of treatment at 13–14° C. with a very large amount of mammalian TSH, at least five times that effective in man on a weight basis, the hatchling thyroid follicles are markedly enlarged.

The results of investigations of temperature effects on reptilian thyroids substantiate the observations on elasmobranchs. Eggert (1936) observed that the thyroids of lizards kept at 6–7° C. during the summer are not activated by TSH, whereas at normal summer temperatures, the pituitary hormone is very effective. Shellabarger *et al.* (1956), working with turtles, found that TSH caused increased I^{131} uptake at 21–23° C., but not at 2–3° C. The body temperature of birds and mammals, whose thyroids are more responsive, is much above even the peak temperature in all these instances.

Of course, other factors have to be considered once one leaves the realm of development, such as light intensity and periodism, osmotic conditions, seasonal cycles, degree of maturity, sexual activity (Eggert, 1936; Bileau, 1956; Shellabarger *et al.*, 1956; Hickman, 1959; Leloup and Fontaine, 1960; Olivereau, 1960). The absence of thyroid hypertrophy and hyperplasia that Olivereau (1952) reports for adult *Scyliorhinus canicula* at 20° C. after prolonged treatment with thiouracil could be due to normal lack of utilization of thyroid hormone at the season of the year (autumn) or stage of life, or even under the prevailing conditions of light. Tinacci's (1947) work with *Mustelus laevis* was carried on during the summer, which seems to be a time of high thyroid activity in poikilotherms (Eggert, 1936; Bileau, 1956; Baggerman, 1957).

It may be objected that Dent and Dodd (1961) observed thyroid response to TSH in the spotted dogfish during the winter. Their specimens, however, were hatchlings. Zezza (1937) points out that the thyroid gland of *Torpedo ocellata* presents a much more active histological picture during growth than in adulthood, and the same thing probably holds true for *Scyliorhinus canicula*. At any rate, the gland is very active during development.

The rate of I^{131} removal in Series III at room temperature shows an excellent correlation with both whole-body uptake and thyroid autoradiography. Specimen 15C, with an arithmetic decrease in I^{131}, must have had a constant turnover rate of thyroid iodine and consequent active excretion after exposure to an excess of iodide. On the other hand, in the three goitrogen-treated specimens (3P, 16P, and 17T), diffusion seems to have been the principal mechanism, for iodine removal occurred almost geometrically. Probably the deviation from exact exponential change is due to the fact that the medium remained unchanged and I^{131} could diffuse back into the animals. Specimen 16P, with the lowest uptake, would be most affected by re-entrance of I^{131} from the medium, and would reach equilibrium sooner. Unfortunately, water samples were not counted.

The selachian embryo is a very satisfactory experimental animal, as Vivien (1954) has pointed out. Not only the oviparous forms, such as *Scyliorhinus canicula*, but also the ovoviviparous series, *e.g.*, *Squalus suckleyi* (Pritchard and Gorbman, 1960) can be reared in the laboratory.

Many problems concerning thyroid activity in poikilotherms remain unsettled. Perhaps the study of the embryo, which only gradually develops its homeostatic mechanisms, would reveal the basic function of the thyroid gland, which is then modified as development proceeds and environmental response becomes more complex.

This investigation was supported in part by a PHS research grant, A-1766, from the National Institute of Arthritis and Metabolic Diseases, Public Health Service. The experimental portion of the work was carried out during the tenure of a National Science Foundation Faculty Fellowship, 69021, at the Laboratory of the Marine Biological Association of the United Kingdom, Plymouth, England. I am especially indebted to Dr. F. S. Russell, Director of The Laboratory, and to Dr. T. I. Shaw and Dr. D. B. Carlisle, under whose direction the experiments were performed.

SUMMARY

1. Embryos of *Scyliorhinus canicula,* removed from their cases and kept at either room temperature or 8° C., were treated with 0.05% thiourea or perchlorate, or they were untreated, for 11, 13, or 21 days previous to addition of I^{131} to their medium. After two days of exposure to I^{131} and a day of washing, whole-body counts were made. The thyroid glands were removed within four days and prepared for autoradiography.

2. At 8° C., although the film indicated the presence of a slight amount of bound iodine in the controls, there was no marked difference in whole-body count in the three groups.

3. At room temperature, the controls had very high counts and well-blackened autoradiograms, whereas the two experimental groups had low counts and no sign of organic iodine.

4. Only the experimentals which were exposed to the goitrogens for 28 days showed histological responses, such as enlarged follicles and very numerous mitotic figures.

5. Growth was inhibited at the low temperature, but apparently unaffected by either of the goitrogens at either temperature.

LITERATURE CITED

ADAMS, A. ELIZABETH, 1946. The effects of thiourea on the thyroids of *Triturus viridescens. Anat. Rec.,* **94**: 532.

BAGGERMAN, BERTHA, 1957. An experimental study of the timing of breeding and migration in the three-spined stickleback (*Gasterosteus aculeatus* L.). *Arch. Néerl. Zool.,* **12**: 105–318.

BILEAU, SISTER M. CLAIRE, 1956. The uptake of I^{131} by the thyroid gland of turtles after treatment with thiourea. *Biol. Bull.,* **111**: 190–203.

BOWIE, D. J., AND V. R. EDMONSON, 1960. Gallocyanin as a nuclear stain in autoradiography. *Stain Technol.,* **35**: 1–4.

DALES, S., AND W. S. HOAR, 1954. Effects of thyroxine and thiourea on the early development of chum salmon. *Canad. J. Zool.,* **32**: 244–251.

DENT, J. N., AND J. M. DODD, 1961. Some effects of mamalian thyroid stimulating hormone, elasmobranch pituitary gland extracts and temperature on thyroidal activity in newly hatched dogfish (*Scyliorhinus caniculus*). *J. Endocrinol.,* **22**: 395–402.

DENT, J. N., AND W. G. LYNN, 1958. A comparison of the effects of goitrogens on thyroid activity in *Triturus viridescens* and *Desmognathus fuscus. Biol. Bull.,* **115**: 411–420.

Dimond, Sister Marie Therese, 1954. The reactions of developing snapping turtles, *Chelydra serpentina serpentina* (Linné), to thiourea. *J. Exp. Zool.,* **127**: 93–116.

Eggert, B., 1936. Zur Morphologie und Physiologie der Eidechsen-Schilddrüse. II. Über die Wirkung von hohen und niedrigen Temperaturen, von Thyroxin und von thyreotropem Hormon auf die Schilddrüse. *Zeitschr. wiss. Zool.,* **147**: 537–594.

Ford, E., 1921. A contribution to our knowledge of the life-histories of the dogfishes landed at Plymouth. *J. Mar. Biol. Assoc.,* **12**: 468–505.

Hickman, C. P., Jr., 1959. The osmoregulatory role of the thyroid gland in the starry flounder, *Platichthys stellatus. Canad. J. Zool.,* **37**: 997–1060.

Leloup, J., and P. Fontaine, 1960. Iodine metabolism in lower vertebrates. *Ann. N. Y. Acad. Sci.,* **86**: 316–353.

Lynn, W. G., and J. N. Dent, 1957. Phenylthiourea treatment and binding of radioactive iodine in the tadpole. *Biol. Bull.,* **113**: 160–169.

Lynn, W. G., and J. N. Dent, 1961. A comparison of the responses of *Triturus* and *Desmognathus* to thyroid-stimulating hormone administration. *Biol. Bull.,* **120**: 54–61.

Lynn, W. G., and Annie M. Peadon, 1955. The role of the thyroid gland in direct development in the anuran, *Eleutherodactylus martinicensis. Growth,* **19**: 263–286.

Mitskevich, M. S., 1957. Glands of internal secretion in the embryonic development of birds and mammals. Published for the National Science Foundation and the Department of Health, Education and Welfare, U. S. A., by the Israel Program for Scientific Translations, 1959. 304 pp.

Olivereau, Madeleine, 1952. Action de divers antithyroïdiens sur la destinée de l'iode radioactif ^{131}I dans la glande thyroïde d'un Sélacien (*Scyllium canicula* L.). *C. R. Soc. Biol. (Paris),* **146**: 569–570.

Olivereau, Madeleine, 1960. Quelques aspects anatomiques et physiologiques de la glande thyroïde chez les poissons. *Ann. Soc. Zool. Belg.,* **90**: 83–98.

Pitt-Rivers, R., and J. R. Tata, 1959. The Thyroid Hormones. The Pergamon Press, London. 247 pp.

Pritchard, A. W., and A. Gorbman, 1960. Thyroid hormone treatment and oxygen consumption in embryos of the spiny dogfish. *Biol. Bull.,* **119**: 109–119.

Shellabarger, C. J., A. Gorbman, F. C. Schatzlein and D. McGill, 1956. Some quantitative and qualitative aspects of iodine-131 metabolism in turtles. *Endocrinology,* **59**: 331–339.

Steedman, H. F., 1957. Polyester wax: a new ribboning embedding medium for histology. *Nature,* **179**: 1345.

Tinacci, F., 1947. L'azione de alcune sostanze antitiroidee (tiuracile, metiltiuracile e aminotiazolo) sul *Mustelus laevis. Pubbl. Staz. Zool. Napoli,* **21**: 124–131.

Vivien, J. H., 1954. Quelques exemples de l'utilisation du germe et de l'embryon de Sélacien dans les recherches expérimentales concernant la régulation, les paragénèses, l'organogénèse et la physiologie embryonnaire. *Arch. Anat. Histol. et Embryol. Strasbourg,* **37**: 163–174.

Vivien, J., and R. Rechenmann, 1954. Etude sur la fonction thyroïdienne de l'embryon de Sélacien. *C. R. Soc. Biol. (Paris),* **148**: 170–172.

Zezza, P., 1937. Tiroide, maturità sessuale e gestazione in *Torpedo ocellata. Boll. Soc. Ital. Biol. Sper.,* **12**: 74–76.

RESISTANCE OF THE PURPLE SEA URCHIN TO OSMOTIC STRESS[1]

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The western purple sea urchin, *Strongylocentrotus purpuratus,* is briefly exposed to drastic environmental changes during the very low tides which occur in summer and winter along the central California coast. On such occasions these animals may be subjected to dehydration, due to high temperature, direct exposure to the sun, and particularly the wind. During heavy rains at low tides they may be exposed to considerably diluted sea water. The sea urchin is thus exposed to a wide range of salinities in the intertidal area. The osmotic tolerance of these animals was therefore determined by observations and experiments reported in this paper. During winter months when tide-pool water is diluted by rains, spawning may be observed; therefore, the influence of salinity changes on fertilization and development was also examined. The results reported in this paper suggest that sea urchins and their developing embryos can tolerate considerably greater variations in osmotic conditions than they are likely to meet in their environment, indicating an ample "safety factor" in their constitution.

MATERIALS AND METHODS

The sea urchins were collected at low tides, primarily near Yankee Point, five miles on the Pacific Coast south of Carmel, California. At this point large populations of the purple sea urchin are exposed at low tides. Other collections were made at Moss Beach, California, near Stanford University. At the Hopkins Marine Station the urchins were put into aquaria with running sea water at a temperature varying between 12 and 16.7° C. At the University they were kept in a constant temperature room at 13° C. in well-aerated sea water, changed daily, until the specimens were used.

The resistance of the adult sea urchins to changes in salinity had to be assessed by their reactions and appearance. Healthy sea urchins, if not too crowded, tend to crawl up on the sides of aquaria rather than stay at the bottom, while unhealthy animals usually remain on the bottom. Healthy urchins eat algae avidly, sickly ones do not. Healthy urchins, when overturned, right themselves in a coordinated manner within about a minute, whereas unhealthy ones may fail to do so, or take more time. Stimulation of normal animals with a probe or a bright spot of light (*e.g.,* from an American Optical Company Universal Illuminator) brings about a positive reaction (local erection, in the direction of stimulation, of spines and pedicellariae) and stronger stimulation elicits an escape reaction. These responses are abnormal in unhealthy animals. Finally, animals which are sick or dying

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lose pigment and spines. Detection of slight changes in appearance or behavior of these animals is somewhat subjective, but marked changes are very definite and easy to note. Failure to show any response to prodding indicates that an animal is, to all intents and purposes, dead.

It proved impossible to develop a consistently reliable source of running sea water at various salinities because of difficulties with regulating valves and air embolism in the tubes. The various solutions to be tested were made up in 7-liter battery jars, using appropriate mixtures of sea water, tap water and saturated brine (from the salt pools at Moss Landing, California), and aerated vigorously. Eight to 10 animals, 3.0–3.5 cm. in test diameter and totaling about 200 grams wet weight, were kept in each jar. The concentrations of sea water tested were 61.5%, 70.4%, 80.7%, 90.3%, 110%, 121.4%, 130.8%, 150% and 170% as determined by titration with silver nitrate, using potassium chromate as indicator.

In the developmental studies at different salinities the following procedures were observed: Ovaries were removed from gravid female sea urchins, avoiding contamination with other tissues, and suspended in filtered sea water in Syracuse watch glasses. The concentrated egg suspension was picked up in a mouth pipette under a dissecting microscope and added, with a minimum of sea water, to about 1 ml. of the appropriate hypertonic or hypotonic sea water (made up with distilled water). Sperms were added in small quantities from the tip of a needle, and the progress of fertilization, first and second cleavages, early and late blastulation, gastrulation, and in some instances, pluteus formation, was noted.

Changes in the osmotic pressure of the perivisceral fluid of the sea urchins exposed to sea water of various tonicities were determined by the method of Gross (1954). The effects of tonicity changes of sea water on the respiration of sea urchins were studied with the standard Warburg-Barcroft manometric method, using large respirometric flasks which readily accommodate small sea urchins.

In all experiments small sea urchins were employed in order to have a more homogeneous population sample. For respirometry they had to be 2–3 cm. in diameter to fit into the flask (Farmanfarmaian, 1959). In the other experiments specimens 3.0–5.5 cm. in diameter were used. Experiments were carried out soon after the animals were collected and when they were all well fed and healthy. Feeding was avoided during experiments (except when testing the feeding reaction) to prevent fouling the aquaria. The latter had to be cleaned frequently at first because of extensive defecation. When an animal died it was promptly removed, since all the other animals in a container will soon die unless this is done.

Experimental Results

1. *Osmotic tolerance of sea urchins*

It is apparent from the results in Table I that the extreme salinities are almost immediately injurious; no response to stimulation was obtained after a three-hour exposure of sea urchins to 30%, 50%, 150% and 170% sea water. Those in 60% and 130% sea water, which also showed no response after three hours, recovered when replaced in sea water. Most of the studies were concerned with the other concentrations, close to sea water, because they lie within the range of greater ecological interest.

TABLE I

Osmotic tolerance of adult Strongylocentrotus purpuratus*

Per cent sea water	Effect of short exposure (3 hrs.)	Effect of prolonged exposure (days)
30	No activity or response; no recovery (die)	—
50	No activity or response; recover later**	Die by second day.
60	Little activity or response; recover later**	Lose much pigment; unresponsive; most of them die by the second day.
70	Normal at beginning	Stay near bottom of container; some die on 5th day, all by 25th day.
80	Normal***	Lose some pigment for first three days; survive†—35 days.
90	Normal***	Lose a little pigment for first three days; survive†—35 days.
100	Normal***	Normal; survive†—35 days.
110	Normal***	Lose some pigment continuously; some reduction in activity; survive†—35 days.
120	Normal at beginning, reduced response; recover later**	Lose pigment, stay near bottom of container; some dead by 7th day; half are dead by the 35th day.
130	Little activity or response; recover later**	Lose pigment; die on second day.
150	No activity or response; no recovery	—
170	No activity or response; no recovery	—

* Temperature varied between 12.0 and 16.7° C. during the course of these experiments.

** When replaced into sea water (100%).

*** For the entire period of observation—35 days.

† Climb up sides of container (at least at first); right themselves rapidly; feed upon *Iridaea* (red alga); respond to bright light and touch.

Although the sea urchins tolerated 70% and 120% sea water for three hours, they were not normal after a more prolonged exposure and they stayed near the bottom of the tank instead of climbing along the sides as did the controls. They then lost pigment and many died in 70% and 120% sea water between the twenty-fifth and thirty-fifth day of exposure. These concentrations of sea water, therefore, constitute the limits of tolerance. Tonicities of 80%, 90% and 110% sea water were tolerated, the animals remaining essentially normal for at least 35 days (in some cases to 50 days) of exposure and observation. Needless to say, controls in sea water remained normal for much longer periods.

The response to changes in the concentration of sea water is tolerance and not regulation. This is indicated by the change in weight of sea urchins following immersion in sea water of diverse tonicities, as seen in Figure 1A for one series of experiments. The animals used were 3.0 to 5.0 cm. in test diameter and weighed 25 to 35 grams. They were immersed in 500 ml. of the appropriate solution in a 600-ml. beaker and drained on a towel for 5 minutes before weighing. The change in weight was calculated as per cent of original weight. Figure 1B shows the change in the osmotic pressure of the perivisceral fluid as a result of immersion

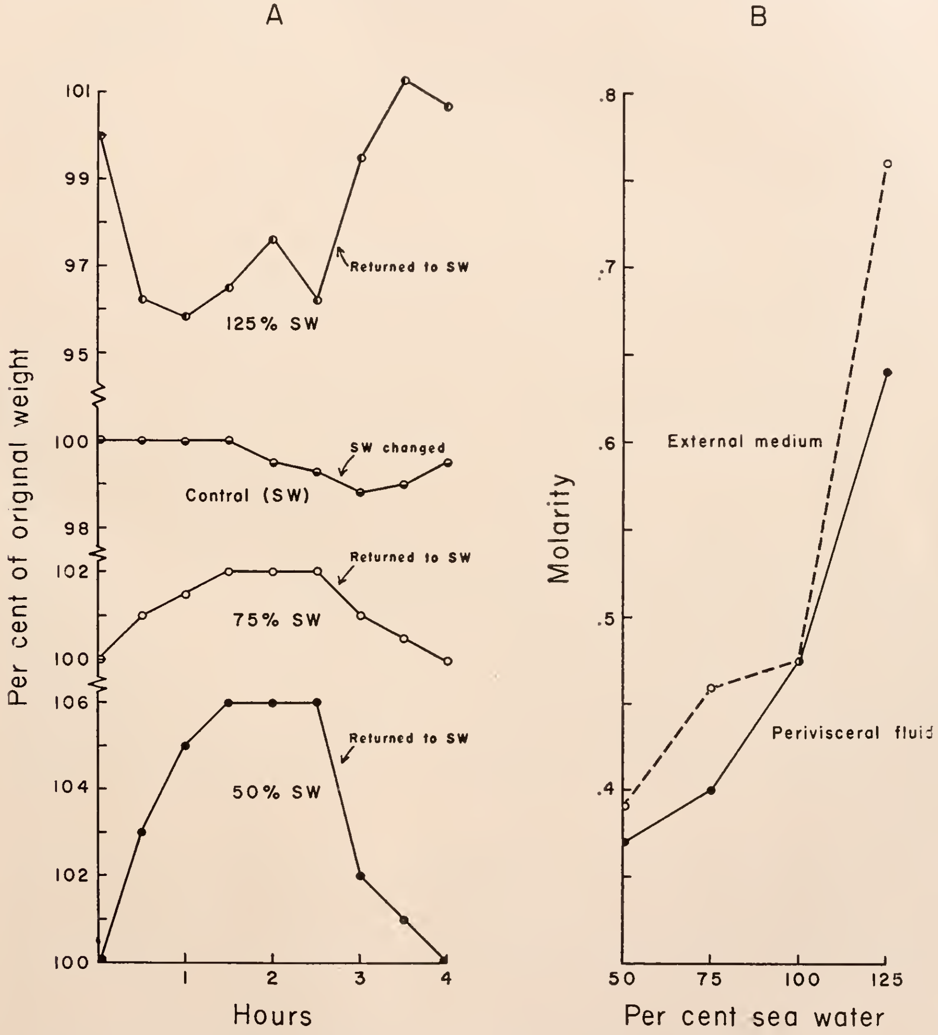

FIGURE 1. A. Changes in weight of sea urchins placed in various concentrations of sea water and again after replacement in sea water. B. Changes in concentration of perivisceral fluid at equilibrium (after a 1.5-hour exposure of the sea urchin to the solution); concentration is given in terms of NaCl equivalents. Note that the perivisceral fluid approximates the external bathing medium in concentration.

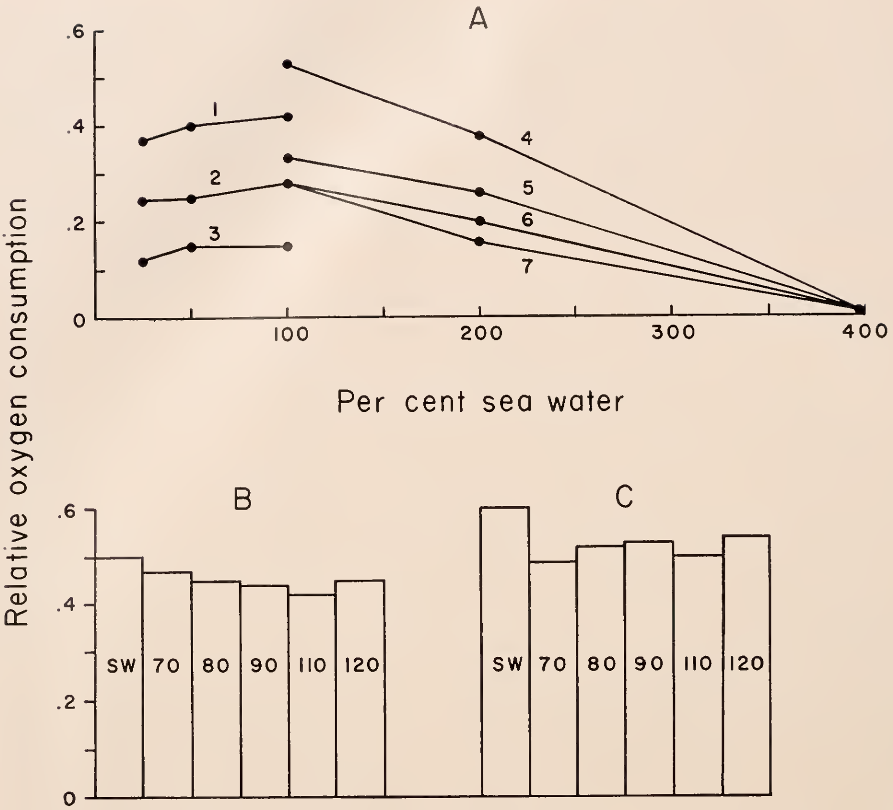

FIGURE 2. A. Relative change in oxygen uptake of sea urchins placed in various concentrations of sea water. The changes in respiration at various concentrations of sea water were studied in the order indicated, starting with each sea urchin in normal sea water. The initial oxygen consumption recorded is characteristic for each sea urchin, depending upon its size and other characteristics. B and C. Histograms of changes in oxygen consumption of two sea urchins with changes in concentration of sea water within the range of tolerance. Similar results (omitted here) were obtained with three other specimens. In all cases, slight changes in oxygen uptake were observed with variations in concentration of the bathing sea water. The initial oxygen uptake of each specimen depends upon its size and other characteristics. Comparisons should be made for the same sea urchin at different concentrations of bathing sea water.

of the urchins in sea water of diverse tonicities. In each test the capillaries used for the melting point tests were partially filled with either the bathing fluid or the perivisceral fluid. The latter was obtained by inserting the capillary directly through the peristomial membrane into the main coelom. Care was taken not to contaminate such samples with the bathing fluid. The results in Figure 1B show that the osmotic pressures of the perivisceral fluid and external media are about the same once equilibrium is established, *i.e.*, no change in weight is observed. The differences observed at 75% and 125% sea water are probably due to incomplete equilibrium.

Animals which regulate the salt concentration of their body fluids expend metabolic energy and often increase their metabolic rate under conditions of osmotic stress (Prosser and Brown, 1961). The respiration of sea urchins exposed to various salinities was measured, to determine whether changes in salinity altered the respiratory rate. The respiratory rate for each individual was first measured in normal sea water. This water was then aspirated and replaced by the test solution, and determinations were started only after 40 minutes of equilibration. In longer experiments one or more controls were used to safeguard against possible changes due to internal clocks or other uncontrollable factors. The values obtained for experimental animals were then corrected by the changes in these controls (for methods, see Farmanfarmaian, 1959).

The data shown in Figure 2A for three series of experiments indicate that there is no marked change in respiration even when the urchins are immersed in 50% sea water, and only a small decrease when they are in 25% sea water, and most marked in 400% sea water.

Within the tolerance range (70% to 120% sea water) the variation in respiration is indeed very slight as shown by 5 series of experiments. Only two of these experiments are shown in Figures 2B and C.

To determine whether the osmotic stress of diluted sea water could be relieved by addition to the medium of an inert, non-penetrating organic compound, sea urchins were subjected to 50%, 25% and 5% sea water made equivalent to sea water in tonicity by the addition of one molal sucrose (A. Klein and R. Rasmussen, unpublished data), much in the manner of Loeb's study on a crustacean in 1903. After two hours the sea urchins appeared dead by the response criteria (see Methods). Tests of the body fluid chlorinity by silver nitrate titration (using silver chromate as indicator) showed some loss of salts from the body fluid. Tests for reducing sugar in the body fluid (after hydrolysis with HCl) were negative, indicating no marked entry of sucrose (the method would not have detected slight entry). Sea urchins, after two hours in each of these media, replaced in aerated sea water responded and appeared normal after 24 hours. At this time body fluid osmotic pressure (method of Gross, 1954) was normal and the weight, which had initially dropped, was essentially back to normal (slightly greater). When sea urchins were placed directly in one molal sucrose, irreversible damage occurred within two hours. Tests now disclosed more rapid loss of salts and marked entry of sucrose, suggesting that the cells lining the sea urchin membranes had died, and that equilibrium was being established between the body fluid and the external medium.

The experiments do not tell what is happening to the cells of sea urchins placed in diluted media. Perhaps salt losses in them are much quicker and more marked than can be detected in the massive body fluid which was tested, and loss of excitability may well follow such cellular depletion. In any case, sucrose protects against dilution of sea water to only a limited degree, indicating that the problems which the sea urchin meets in media of altered osmotic pressure are ionic as well as osmotic.

2. *Osmotic tolerance of sea urchin eggs and developmental stages*

The results of tonicity changes on the development of sea urchin eggs are shown in Table II. Eggs immersed in 200% sea water perceptibly crinkled (due to loss

TABLE II

Osmotic tolerance of developmental stages of Strongylocentrotus purpuratus

Per cent sea water	Fertilization membrane	Cleavage* to 2 cells	Cleavage* to 4 cells	Blastulae*	Gastrulae*
50 + 0.5 *M* sucrose	tight	normal	normal	almost like control	almost like control
60	about half; eggs swell	occasional, abnormal, delayed	abnormal delayed	abnormal, multicellular masses	—
70	all eggs** swell	much like 80% but more exaggerated	delayed	much delayed and abnormal	—
80	all	much like controls, blastomeres rounded	delayed	delayed, but good	abnormal fragmenting
90	all	much like controls	seemingly normal	like control	like control
100	all	normal	normal	normal	normal
110	all	most cleave	seemingly normal	like control	like control
120	all	most cleave, delayed	delayed	delayed, small	many quite abnormal
150	about a fifth; sperms active	a few cleave	delayed	some blastulae***	—
100 + 0.5 *M* sucrose	sperms immobilized	some cleave	delayed	abnormal	—
200	none; sperms immobilized; eggs shrink	—	—	—	—

* The time table for cleavage of eggs of *S. purpuratus* at 13° C. has been given elsewhere (Giese, 1938). The first cleavage requires almost two hours at 13° C., the subsequent cleavages occur at about hourly intervals. Free-swimming blastulae are found 20 hours after insemination, gastrulation occurs in about 32 hours after insemination, and the gut begins to differentiate in 46 hours after insemination. Plutei form in about 96 hours, although the arms do not appear until about 120 hours after insemination.

** "Like controls"—usually a few eggs in both control and experimental series do not develop fertilization membrane and fail to develop.

*** In one series some abnormal blastulae developed; in one, only abnormal masses of nonmotile cells.

of water), the sperms were immediately immobilized, and failed to penetrate the eggs. Later examination showed no signs of fertilization or cleavage. In 150% sea water the sperms were highly motile and about 20% of the eggs mixed with sperms developed fertilization membranes, but such cleavages as occurred were delayed and quite abnormal. In 120% sea water the eggs fertilized to the extent that controls did, but cleavages were delayed and resulted mostly in abnormal balls of cells. However, 24 hours later some blastulae became motile. Eggs in 60% sea water were also so damaged that only about half of them showed fertilization membranes, and only about 20% cleaved; none formed blastulae. In 70% sea water early cleavage was observed but later cleavages were much delayed and abnormal. The blastomeres of cleaving eggs were more rounded and larger than the controls in sea water. Eggs at the other concentrations (80%, 90%, 100% and 110% sea water) developed normally to the early blastula stage, and 24 hours later all except those in 80% sea water formed swimming blastulae. The majority of the embryos in 80% sea water were definitely unhealthy although a few normal free-swimming blastulae appeared at the same time as in the controls. Those in 90% sea water appeared even healthier than controls in sea water.

Twenty-four hours later, gastrulae appeared in the 110%, 100% and 90% sea water; in one of 8 trials with 80% sea water, gastrulae appeared but they were not normal.

It is interesting, however, that blastulae and gastrulae transferred from the control in sea water to 70%, 80%, 120% and 150% sea water were alive and healthy 8 hours later. Those in 150% sea water were smaller than controls, and each had a shorter gut and a dense mass of cells around it. All survived for a week, at which time the experiment was terminated. At this time controls were in the late prism stage with skeletal rods and well-developed gut just preceding formation of the pluteus. Only the embryos in 110% sea water showed a degree of development comparable to the controls. Those in 90% sea water were almost as well developed but neither the skeletal rods nor the gut were comparable to those in the controls. Those in concentrations far to either side of sea water remained as enlarged and undifferentiated gastrulae, although in some of them a mouth opening appeared. Thus, it is apparent that not only the early development, but also the later development of the embryos is adversely affected by tonicities of sea water much removed from the normal medium.

Changes in the development of sea urchin eggs observed in the hypotonic and hypertonic sea water discussed above are apparently due to changes in osmotic pressure rather than the quantity of the salts. This proposition was borne out by a series of experiments in which 50% sea water was made isosmotic with sea water by addition of approximately 0.5 molal sucrose (see Loeb, 1908; Harvey, 1956). The various stages of development from fertilization to the advanced gastrula are essentially normal in such a solution (see Table II). The rate of development and the percentage of zygotes attaining the advanced gastrula stage also are comparable to controls. It must be stated, however, that embryos developing in such a solution appear to be a little more compact than the controls. Also, when control blastulae are transferred to this sucrose-sea water medium they lose water at first but soon attain equilibrium and appear normal thereafter.

There is a limit, however, to which sucrose may be substituted for the salts of sea water without altering development. Thus, when 5% sea water is made isosmotic by the addition of sucrose, sperms are quickly immobilized and the fertilization rate drops drastically. It is known that changes in the solute environment alter the permeability of cells (Lucké, 1940). Development of the few eggs which are fertilized is completely abnormal. Also normal blastulae transferred to such a solution became abnormal within 6 hours. The minimal salt requirements for normal development of *S. purpuratus* are being studied (Deboyd Smith, unpublished). Extensive studies of this type were made by Herbst (1903) on eggs of European sea urchins.

The effects of a 0.5 molal sucrose solution made up in sea water were similar to those observed in 150% sea water. The sperms were quickly immobilized, and only a small percentage of the eggs were fertilized. The zygotes formed very compact abnormal blastulae, and normal blastulae transferred to the hypertonic solution swam actively for 12 hours but became quite abnormal.

DISCUSSION

Echinoderms are typically marine animals but a few are found in brackish water, for example, *Asterias rubens,* which occurs in the Baltic Sea (Schlieper, 1956). This sea-star may live in a salinity as low as 8 parts per thousand (8‰) in the middle Baltic near Rügen. It can be moved from 8‰ to higher salinities without damage, and from higher salinities to lower ones—but only gradually—permitting progressive adaptation over a period of several weeks. While the sea-stars tolerate such lowered salinities, it is interesting to note that they grow to a smaller size and show changes in many characteristics. For example, in water at 15‰—as compared to 30‰—righting reactions are slower, the gonads develop more slowly (although to the same extent), the tissue metabolism is decreased, and the body consists of more water and less ash. Since at lowered salinities, *Asterias* reaches osmotic equilibrium with the bathing fluid, it is possible that the reduction in internal salt concentration affects the activity of the enzymes in the cells (Schlieper, 1956). There may also be a dilution of the enzymes since the ratio of water to ash increases at lower salinities.

In a study of the fauna of an estuary along the coast of Maine, Topping and Fuller (1942) report that *Strongylocentrotus dröbachiensis* was found only where the salinity was just slightly less than in the sea. There appear to be no records of echinoids as resistant to low salinities as the asteroid *A. rubens.*

S. purpuratus on the California coast is probably exposed to air[3] in shallow pools for two- to three-hour periods a few times a year, mainly at the low tides of winter and summer. The exposure period is too short to allow sufficient evaporation to make the tide-pool water hypertonic. However, exposure to rain at low tides during the winter and spring months (December to April) may result in significant dilution of the sea water.

Dilution is not general, since the local shore water tested over the year varies only slightly (28.7‰ to 34.18‰ at the Hopkins Marine Station, according to Feder, 1956). Considerable dilutions do occur, however, when torrents of land wash pour into isolated sea urchin tide-pools at low tides during and after heavy rainfall. On December 23, 1955, records indicate 2.7 inches of rain within 24 hours at Carmel, California. During the same period there were low tides of 0.0 and minus 0.7 foot magnitude. The sea urchins were exposed to nearly 50% sea water for as long as two to three hours. On February 24, 1958, titrations from similar pools after a rainfall of 0.72 inch (low tide of plus 0.7 foot) indicated a dilution to 72% sea water. It is therefore not surprising that the purple sea urchin is able to survive exposure to variations in the tonicity of sea water and is capable of withstanding, for a few hours, considerable dilution of the surrounding sea water. In nature, exposure to diluted sea water is probably always brief, since mixture with the main sea water mass quickly restores a concentration close to normal.

Because the purple sea urchin spawns on the California coast during the rainy season, legend has associated spawning with dilution of the sea water, although no proof of a causative relationship between the two is available. Spawning, however, does occur during this season and the eggs are fertilized and may develop for periods of time in diluted sea water. Since a small degree of dilution of the sea water does not affect development of the eggs, the rains probably do not critically affect survival of the embryos developed in nature during this time.

It might be argued that the resistance of developmental stages of *S. purpuratus* to dilution (or concentration) of sea water represents an adaptation enabling the sea urchin to survive the changes in tonicity it meets in its environment. A similar resistance of the eggs of the echiuroid worm, *Urechis caupo*, to variations of concentration of sea water, comparable to what may occur in its native habitat, has also been recorded (Giese, 1954) and might also be considered an adaptation permitting survival. However, some unpublished studies with the eggs of the deep sea urchin, *Allocentrotus fragilis*, indicate that development in this species is about as resistant to dilution or concentration of the sea water as is *S. purpuratus*. Thus, the delay in cleavage of *A. fragilis* eggs caused by variation in concentration of sea water is about the same as that described above for *S. purpuratus*, and delayed blastulae developed at 150%, 120%, 80% and 70% sea water, becoming progressively fewer in number and more abnormal the greater the deviation from sea water. Normal blastulae, comparable to controls in 100% sea water, were obtained in 90% and 110% sea water. Yet *A. fragilis* lives in deep water (Boolootian *et al.*, 1959) where appreciable changes in salinity are not recorded (Sverdrup *et al.*, 1942). It must be remembered, however, that the larvae of this organism are pelagic (Moore, 1959) and may possibly be exposed to significant variations in salinities since they breed in winter (Giese, 1961) when rains are heavy. However, tolerance, by gametes and other cells of the purple sea urchin, of changes in the osmolality of the medium is perhaps only a measure of the general tolerance of such changes by cells of most marine organisms.

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[3] A. Klein and R. Rasmussen (unpublished) found an appreciable loss in weight and an increase in osmotic pressure of the body fluid of sea urchins exposed to air for 24 to 48 hours.

SUMMARY

1. The west coast purple sea urchin, *Strongylocentrotus purpuratus*, was found to be resistant to dilutions and concentrations of sea water for a brief time (three hours) within the range 70% to 120% sea water.

2. The sea urchins resist exposure for many days (35) in 80% to 110% sea water, inclusive. Damage at higher and lower concentrations of sea water is indicated by loss of activity, loss of pigment and appendages, and failure to respond to food, probing, and light.

3. The sea urchins lose weight in hypertonic solutions and gain weight in hypotonic solutions, presumably losing and gaining water, respectively. They recover nearly normal weight after replacement in sea water.

4. The respiration of the sea urchins is little altered by changes in tonicity of the sea water, except at extremes of tonicity where the metabolic rate is decreased.

5. Sea urchin eggs develop normally over the range 90% to 110% sea water, and considerable development occurs over the range 70% to 150% sea water.

6. Gastrulae placed into various concentrations of sea water tolerate the change no better than developing eggs; differentiation proceeds in 90% to 110% sea water, but is essentially stopped at higher or lower concentrations even though the gastrulae survive.

7. Addition of sucrose to 50% sea water to make it to up to 0.5 molal resulted in a medium as favorable as sea water to early cleavage and early development of the sea urchin egg. Addition of sucrose to sea water to make it up to 0.5 molal was as deleterious as 150% sea water to cleavage and early development of the sea urchin egg.

8. Relating the results obtained with sea urchin embryos and adults, it is apparent that the sea urchin is capable of withstanding the maximal variations in the salinity of its natural environment.

LITERATURE CITED

BOOLOOTIAN, R. A., A. C. GIESE, J. S. TUCKER AND A. FARMANFARMAIAN, 1959. A contribution to the biology of a deep sea echinoid, *Allocentrotus fragilis* (Jackson). *Biol. Bull.*, **116**: 362–372.

FARMANFARMAIAN, A., 1959. The respiratory surface of the sea urchin, *Strongylocentrotus purpuratus*. Ph.D. Thesis, Stanford University.

FEDER, H., 1956. Natural history studies on the starfish *Pisaster ochraceus* (Brandt, 1835) in the Monterey Bay area. Ph.D. Thesis, Stanford University.

GIESE, A. C., 1938. The effect of ultraviolet radiation of λ 2537A upon cleavage of sea urchin eggs. *Biol. Bull.*, **74**: 330–341.

GIESE, A. C., 1954. Osmotic tolerance of the developing *Urechis* egg. *Anat. Rec.*, **120**: 768.

GIESE, A. C., 1961. Further studies on *Allocentrotus fragilis*, a deep sea echinoid. *Biol. Bull.*, **121**: 141–150.

GROSS, W. J., 1954. Osmotic responses in the sipunculid, *Dendrostomum zostericolum*. *J. Exp. Biol.*, **31**: 402–423.

HARVEY, E. B., 1956. The American Arbacia and Other Sea Urchins. Princeton University Press, Princeton, N. J.

HERBST, C., 1903. Ueber die zur Entwicklung der Seeigellarven notwendigen anorganischen Stoffe, ihre Rolle und ihre Vertretbarkeit. III. Theil. Die Rolle der anorganischen Stoffe. *Arch. f. Entw.*, **17**: 306–520.

LOEB, J., 1903. On the relative toxicity of distilled water, sugar solutions and solutions of the various constituents of sea water for marine animals. *Publ. Univ. Calif. Physiol.*, **1**: 55–69.

LOEB, J., 1908. Qu'est-ce qu'une solution de saccharose isotonique pour les oeufs *Strongylocentrotus? C. R.. Acad. Sci. Paris*, **146**: 246–249.

LUCKÉ, B., 1940. The living cell as an osmotic system and its permeability to water. *Cold Spring Harbor Symp.*, **8**: 123–132.

MOORE, A. R., 1959. On the embryonic development of the sea urchin *Allocentrotus fragilis*. *Biol. Bull.*, **117**: 492–496.

PROSSER, C. L., AND F. A. BROWN, JR., 1961. Comparative Animal Physiology. Second ed. Saunders, Philadelphia.

SCHLIEPER, C., 1956. Comparative study of *Asterias rubens* and *Mytilus edulis* from the North Sea and the western Baltic Sea. *Ann. Biol.*, **33**: 117–127.

SVERDRUP, H. V., M. JOHNSON AND R. FLEMING, 1942. The Oceans. Prentice Hall Inc., New York.

TOPPING, F., AND J. FULLER, 1942. The accommodation of some marine invertebrates to reduced osmotic pressures. *Biol. Bull.*, **82**: 372–384.

FACTORS OF EQUATORIAL CONTRACTION AND POLAR MEMBRANE EXPANSION IN ENDOSPERM CYTOKINESIS

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In a recent review (Roberts, 1961) I suggested that cytokinesis in both plant and animal cells might be explained in terms of the factors of polar expansion, equatorial contraction, and new membrane formation. Mazia (1961) has also discussed basic similarities in cytokinesis of plant and animal cells. There is considerable support for the idea that new membrane formation may occur in the connecting stalk of dividing animal cells late in cleavage (references and discussion in Roberts, 1961; Mazia, 1961; Buck and Tisdale, 1962a, 1962b). Buck and Tisdale (1962a) have demonstrated in rat erythroblasts the formation of a midbody in the equator of the spindle during cleavage, which appears to correspond in structure to the phragmoplast of plant cells, and (1962b) have described in three types of mammalian cells membrane-bound vesicles, apparently derived from endoplasmic reticulum, which develop in the cleavage plane and apparently are the source of new cell membrane in the furrow region. Their observations are somewhat similar to those of Porter and Machado (1960) on cell-plate formation in *Allium*. However, there has been almost no evidence that polar expansion and equatorial contraction might play a part in cytokinesis of plant cells which employ cell-plate formation, other than the observations of Bajer and Molè-Bajer (1956) on the division of well flattened living endosperm cells. *"The lack of cellulose walls makes possible the formation of cytoplasmic protuberances. These pseudopodia-like strings of cytoplasm may form during the whole division, but are most prominent in prophase and telophase. During pro- and metaphase the shape of the cell often changes to a sphere or an ellipsoid—often without pseudopodia formation. It is of interest that in other material the cells often become round before metaphase. In animal tissue it is well known that pseudopodia disappear before anaphase. . . . Endosperm cells often form outpushings during the telophase."* These suggestions of surface forces in dividing endosperm, similar to those of dividing animal cells, stimulated the writer to look at dividing living endosperm cells which had not been distorted by flattening.

Investigators are rightfully concerned with standards of normalcy in studies of living cells, and with conclusions based on cells studied under abnormal conditions. Some would reject all studies of cell division based on cells which do not complete at least two divisions without evidence of abnormality. Such a criterion would impose serious and frequently impossible difficulties on the study of terminal divisions, as in secondary spermatocytes, and eliminate as subjects of investigation many cells with long division cycles. Other investigators recognize that degrees of abnormality are the almost inevitable result of preparation and observation. They believe that there is information to be gained from abnormal cells, provided the investigator recognizes and makes extremely clear the limits of his methods and of his results, and is extremely cautious in his conclusions. The observations here reported were made on cells which do not meet the criterion above and which were dividing under abnormal conditions. The limitations are detailed in the body of the paper and are considered in the discussion. Subject to these limitations the observations support the idea that equatorial contraction and polar expansion may play a significant role in cytokinesis of endosperm cells lacking a cell wall.

Materials and Methods

Young seeds still in the milk stage of whatever horticultural varieties of *Hemerocallis* were available at the moment, were the source of endosperm. Seeds collected at mid-day or early afternoon provided the best material. Those collected in the early morning contained few dividing cells. One end of the seed was sliced off with a sharp scalpel, and endosperm was sucked out of the cavity with a 1-ml. syringe fitted with a 24-gauge needle whose tip had been ground to roundness. The seeds were not squeezed to express more material. Four to eight seeds provided enough material for a preparation. A 5–7-mm. square was outlined on a no. 1 coverslip with Vaseline extruded through a 24-gauge hypodermic needle, and the enclosed space flooded with endosperm fluid. Excess fluid was carefully withdrawn with the syringe, leaving a relatively flat film whose depth could be adjusted with some experience. The coverslip was then inverted over the shallow depression of a Fisher-Littman well slide and sealed by a ring of Vaseline. Preparations were examined and photographed by phase contrast microscopy using a 12.5 × compensating ocular and 45 × dark contrast objective. The microscope (Spencer) was equipped with long focal length phase plates. Illumination was provided by a ribbon filament lamp with 36 mm. of 3% copper sulfate solution in the light path as a heat absorber. Light intensity was controlled by a variable transformer and was kept at the minimum possible for observation except for the few seconds required for photographic exposure.

The preparations differ significantly from those of the Bajers (1954; 1956). Thick hanging drop preparations were necessary to avoid flattening. This introduced problems already described by Bajer (1954, p. 386). ". . . *the thickness of the drops is of greatest importance. In large drops the penetration of oxygen is not sufficient, and in consequence most cells die in prophase, though most mitoses in metakinesis are continued to telophase.*" Bajer found it necessary to use drops only six micra in thickness. Since freely suspended cells were essential a soft agar substrate could not be used. Most of the first season of study was devoted to efforts to achieve longer-lived preparations, without significant success. It was thus necessary to accept the limitations imposed by the requirement for thick preparations and freely suspended cells, and resort to the simple preparations described. Although the small air space of the thin slides used was rapidly saturated, as judged by condensation, evaporation ensured that the medium was at least mildly hypertonic. Preparations thus made were short-lived. After 1½–2 hours the cells showed obvious signs of abnormality and division soon ceased. Cells first observed in prophase could be followed to metaphase. Those first observed in metaphase could be followed to late anaphase. Cells in middle or late anaphase could be followed to telophase and completion of the cell plate.

Routinely each preparation was completely scanned by overlapping traverses and the location of suitable cells for further observation and photography noted. Such scanning was completed in approximately five minutes. It is emphasized that all of the stages described were regularly observed during the preliminary scanning, long before there were evidences of abnormality. The investigation extended through two summers. Seventy cells were followed in detail, and 13 divisions recorded photographically. Incidental observations and photographs were made of many more. Only those cells which continued to divide, subject to the limitations previously described, are the basis of the following observations.

Observations and Discussion

The preparations contained abundant particulate material, ranging in size from barely visible to 1–2 micra, cellular debris, bits of peripheral endosperm tissue with well developed cell walls, and free nuclei. The numerous small particles are a source of difficulty in observing and photographing cells deep in the fluid but

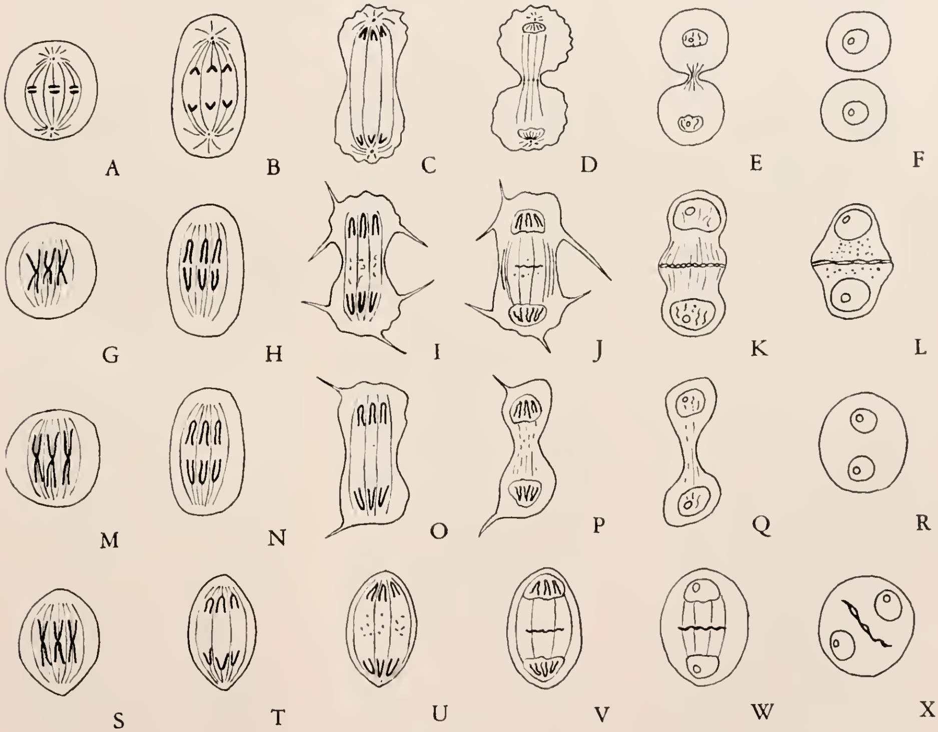
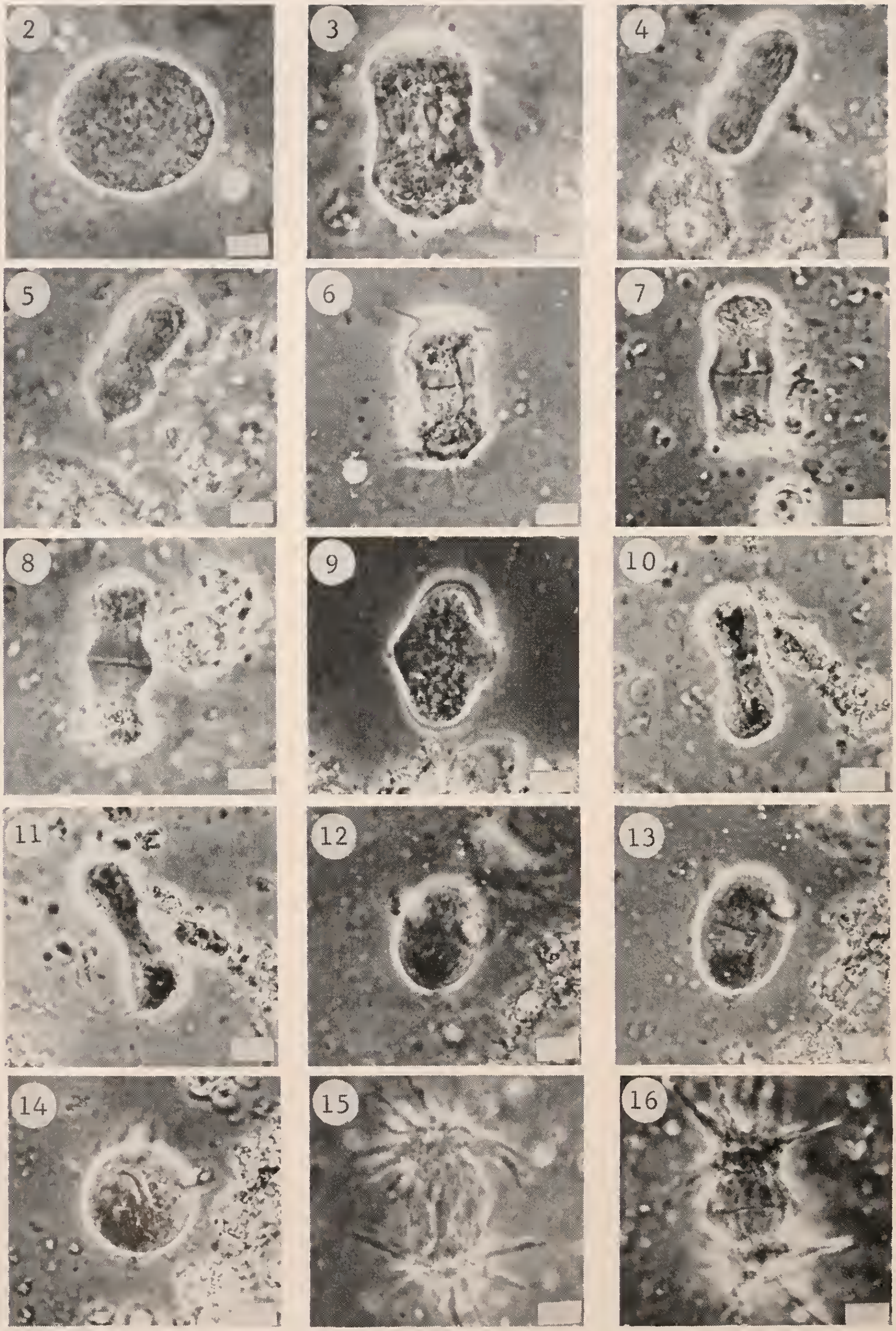


Figure 1. Comparison of cytokinesis of animal cells and endosperm. A-F, cytokinesis of animal cells. In C-D polar membrane expansions, which may take different forms or may be demonstrable only indirectly in various cell types, are shown as surface irregularities. G-L, cytokinesis in endosperm. Equatorial constriction and the two types of surface irregularities are shown in I-J. M-R, failure of cell plate formation and of cytokinesis in endosperm, based on a single cell. S-X, absence of equatorial constriction and polar irregularities followed by failure of cytokinesis.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Figures 2–16.

the presence of the smallest particles in active Brownian movement above the cells being observed ensured that they were not compressed between the coverslip and the surface film. The free nuclei were not observed to divide nor were recognizable prophases of division seen. It is worthy of comment that most of these nuclei had adherent granular material which could not be optically distinguished from the smaller particles seen in the endosperm fluid but lacked visible cell membranes. Division was observed only in *cells* with cytoplasm and enclosed in a cell membrane. The cells, when flattened, were entirely comparable to those studied by the Bajers (1954, 1956) and are believed to be of the same type. Their origin within the endosperm sac is unknown but is presumed to be the same gelatinous layer which the Bajers expressed from the seed.

Figure 1 summarizes the observations on the process of cytokinesis and provides a comparison with similar stages in animal cells. At metaphase the cells round up (Figs. 1G, 2), much as described in the previous quotation from Bajer. During anaphase the cells elongate (Fig. 1H), and in late anaphase or early telophase develop a distinct equatorial constriction (Figs. 1, I–J, 3–5), entirely comparable in appearance to early furrow formation in animal cells. At about the same time irregularities appear in the membrane at the cell poles. These irregularities are of two types; small bulges which appear in the same location and at the same stage of division as the blebs and visible expansions characteristic of cleaving animal cells, and long oriented spinous processes (Figs. 1 I–J, 3–6). Small particles, which have become increasingly visible in the spindle, aggregate in the equator and fuse to form the cell-plate which extends peripherally, eliminating the equatorial constriction and producing a pronounced bulge at the equator (Figs. 1K, 7–8). The polar membrane irregularities gradually disappear, leaving a configuration like that seen in Figures 1K, 7–8. This gradually shortens to the condition seen in Figures 1L, 9, the final stage which could be followed. Except that they are at all times enclosed in a cell membrane, the configurations assumed by the dividing cells are quite comparable to those shown by Jungers (1931).

The similarity to animal cell cytokinesis is striking and is well illustrated in Figure 1. The manner in which such an illustration is drawn can dramatize similarities and de-emphasize differences, but reference to the photographs (Figs. 2–16) should be convincing, especially to those who have observed division of freely suspended animal cells. The observations establish the fact of equatorial

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FIGURES 2–3. Metaphase and anaphase of the same cell. Figure 3 shows equatorial constriction, membrane irregularities and one spinous process. In these and the following figures the scale, 480 ×, is shown by the small rectangles, whose length indicates 10 μ.

FIGURES 4–5. Middle and late anaphase of the same cell, showing development of equatorial constriction and polar irregularities. Comparison with Figures 2–3 indicates range of cell size observed.

FIGURE 6. Cell plate formation in early telophase. The spinous processes are well developed but not unusually so.

FIGURES 7–9. Telophase and the completion of cytokinesis. Figure 9 is the same cell shown in Figure 6.

FIGURES 10–11. Failure of cell plate formation and maximum observed equatorial constriction. In this cell cytokinesis failed and a binucleate resulted.

FIGURES 12–14. Absence of equatorial constriction and membrane irregularities. The cell plate in Figure 14 appeared to be degenerating.

FIGURES 15–16. "Amphiastral" configurations in medium made hypertonic with glucose. The spinous processes suggest astral rays.

constriction but of themselves tell us nothing of its nature. It does not appear to result from conformation of the cell membrane to the contours of the internal spindle and re-forming nuclei. Small particles in Brownian movement may occasionally be seen between the spindle and equatorial constriction, and around the re-forming nuclei. There is evidence the equatorial constriction in animal cells is the result of an equatorial contraction (reviewed in Roberts, 1961). I suggest that the same mechanism may be operative in these endosperm cells, probably to a lesser degree. The observed changes in the polar cell membranes undoubtedly are accompanied by an increase in membrane area at the poles. Their occurrence at the same stage of division as the better known polar expansions of animal cells suggests they may play a similar but perhaps less important role in cytokinesis of endosperm cells. The long oriented spinous polar processes deserve special comment. They are straight, or nearly so, and if the long axes are projected backward, all converge in the re-forming nucleus. Occasionally in routine preparations and more commonly in preparations made more hypertonic by the addition of glucose (Figs. 15–16), the spines develop so abundantly that the cells resemble diagrammatic amphiastral figures. Wilson (1900) described somewhat similar "filose" processes extending from the poles of the elongated polar bodies and early blastomeres of *Cerebratulus*. The centers of "filose" activity were correlated with the position of the spindle poles of the amphiastral spindles. Lima-de-Faria (1958), Östergren (1954), and Östergren, Koopmans and Reitalu (1953) have described astral rays in numerous genera and species of higher plants. They are in agreement that the astral rays are small at metaphase and reach maximum development at late anaphase. Appearance and time of development suggest that the observed spinous processes of endosperm cells may be associated with astral development. However, astral rays have not been observed by the writer and other explanations are possible. Occasionally cells have been observed to divide with only minor polar membrane irregularities, without the development of spinous processes.

In a single instance a cell (Figs. 1 M–R, 10–11) was observed to progress to late anaphase quite normally and then fail to form a cell plate. During early telophase the constriction slowly deepened, advancing further than usual but falling far short of dividing the cell into two (Fig. 11). During telophase the cell suddenly rounded up, resulting in a binucleate. More frequently cells, identifiable in early anaphase by their distinctive outlines (Figs. 1 S–X, 12–14), failed to develop either irregularities of the polar membrane or equatorial constrictions. A cell plate formed quite normally but in all cases failed to contact the equatorial cell membranes. In telophase the cells rounded up and the cell plate showed signs of degeneration. Presumably they too would have formed binucleates, but the life of the preparations was too short to observe the final result. Occasionally binucleate cells were observed during the first minutes of a fresh preparation. It may be coincidental but is probably of significance that in every case in which one of the factors of equatorial constriction, polar membrane expansion, or cell plate formation failed, cytokinesis failed.

Observations such as those reported cannot of themselves establish that equatorial contraction and polar membrane expansions occur and are factors in the mechanism of cytokinesis of normal endosperm cells. The limitations of the methods used are such that the observations reported could be ascribed to the effects of the methods employed. However, it is not without significance that in hundreds of preparations, during two seasons, every cell observed to divide did so as described. All of the stages described have been observed during the first few minutes after preparation. Further, the observations reported were made during the 1½–2 hours before signs of deterioration appeared. On this basis the observations do suggest a strong probability that equatorial contraction and polar membrane expansions occur normally and are significant factors in the mechanism of cytokinesis of endosperm cells.

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Summary

Divisions of living endosperm cells of *Hemerocallis* have been observed in hanging drop preparations where they are not distorted by flattening. Equatorial constriction and polar membrane expansions occur in late anaphase and appear quite comparable to corresponding stages of cytokinesis in animal cells. Failure of equatorial constriction, of polar membrane expansion, or of cell plate formation results in failure of cytokinesis. It is suggested that equatorial contraction and polar membrane expansion may be functionally important in the mechanism of cytokinesis of endosperm cells, as they are in animal cell cytokinesis.

LITERATURE CITED

Bajer, A., 1954. Cine-micrographic studies on mitosis in endosperm. I. *Acta Soc. Botan. Poloniae,* **23**: 383–412.

Bajer, A., and J. Molè-Bajer, 1956. Cine-micrographic studies on mitosis in endosperm. II. Chromosome, cytoplasmic and Brownian movements. *Chromosoma,* **7**: 558–607.

Buck, R. C., and J. N. Tisdale, 1962a. The fine structure of the mid-body of the rat erythroblast. *J. Cell Biol.,* **13**: 109–115.

Buck, R. C., and J. N. Tisdale, 1962b. An electron microscope study of the development of the cleavage furrow in mammalian cells. *J. Cell Biol.,* **13**: 117–125.

Jungers, V., 1931. Figures caryocinetiques et cloisonnement du protoplasme dans l'endosperm d'*Iris*. *La Cellule,* **40**: 293–359.

Lima-de-Faria, A., 1958. Recent advances in the study of kinetochore. Pp. 123–157 in *International Review of Cytology,* Vol. III, G. Bourne and J. F. Danielli, eds. Academic Press, N. Y.

Östergren, G., 1954. Astral rays in normal and chemically disturbed mitoses of higher plants. *Congr. Intern. botan.,* 8^e^ Congr., Paris, 1954, sects. 9–10, pp. 15–16.

Östergren, G., A. Koopmans and J. Reitalu, 1953. The occurrence of the amphiastral type of mitoses in higher plants and the influence of aminopyrin on mitosis. *Botan. Notiser,* **106**: 417–419.

Mazia, D., 1961. Mitosis and the physiology of cell division. Chapt. 2, pp. 77–412 in *The Cell,* Vol. III, J. Brachet and A. E. Mirsky, eds., Academic Press, N. Y.

Porter, K. R., and R. D. Machado, 1960. Studies on the endoplasmic reticulum. IV. Its form and distribution during mitosis in cells of onion root tip. *J. Biophys. Biochem. Cytol.,* **7**: 167–180.

Roberts, H. S., 1961. Mechanisms of cytokinesis, a critical review. *Quart. Rev. Biol.,* **36**: 155–177.

Wilson, C. B., 1900. The habits and early development of *Cerebratulus lacteus* (Verrill). A contribution to physical morphology. *Quart. J. Micr. Sci.,* **43**: 97–198.

ECHOES OF ULTRASONIC PULSES FROM FLYING MOTHS

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It has been firmly established by Griffin, Webster and Michel (1960) that flying bats use echolocation in tracking and capturing flying insects. By this means *Myotis lucifugus* is able to detect insects as small as *Drosophila melanogaster* at distances as great as 50 cm. These authors also used acoustic jamming experiments to show that under these circumstances the bat's clue to the presence of an insect is the echo of its ultrasonic cry rather than the sonic output from the vibrating wings of the prey. However, they reaffirmed the observation made by others (Möhres, 1950; Kolb, 1959; Treat, 1955) that bats at rest and not emitting ultrasonic orientation sounds will respond quickly and accurately to the nearby presence of a buzzing insect.

The ability of *Myotis* to determine the distance, direction, and presumably the size of an object as small as *Drosophila* at a distance of 50 cm. suggests that bats might be able to discriminate many other characteristics of larger prey, such as moths, through the properties of their echoes. Most observers of bat behavior have at one time or another tossed pebbles or other inert objects to feeding bats. In the field bats will commonly detect and track such objects, but rarely attempt to attack or capture them. Under laboratory conditions bats can be trained to track and capture non-flying objects such as mealworm larvae tossed in the air (Webster, 1963), but under natural conditions it seems unlikely that they ever encounter potential prey that is not flapping its wings. Although the wing sound seems to be unimportant as a clue (Griffin, Webster and Michel, 1960), it seems possible that the wing movement of the prey may modify the echoes returning to a flying bat and enable it to discriminate a flying insect from a pebble. This possibility was first pointed out by Griffin (1958).

Further indirect evidence in favor of this hypothesis is the often-made observation (*e.g.,* Treat, 1955; Roeder, 1962) that "freezing" is one of the many types of response made by moths to the proximity of bats or when exposed to ultrasound. Under field conditions some moths (and lacewings) cease all flight movement and drop to the ground when exposed to a source of ultrasonic pulses. In one sense this maneuver might be expected to simplify the bat's task of tracking its prey, since the tracker is presented with a target falling in a roughly ballistic and therefore more predictable trajectory compared with a moth's flight path. Since "freezing" behavior can be presumed to have some survival value to the moth, this disadvantageous aspect (to the moth) is possibly offset by the attainment of some degree of acoustic concealment. In passing, it should be pointed out that exposure to a series of bat-like sounds just as frequently causes moths to make a variety of violent evasive maneuvers or to fly directly away from the source (Roeder, 1962). It is the age-old question of whether it is best to duck.

dodge, or run, to which the answer seems to be that there is survival value in variety.

At present there seems to be no direct way of finding out how much information a bat obtains from echo fluctuations produced by the wing movements of its prey. In the following experiment the bat was replaced by a source of ultrasonic pulses and a microphone in order to determine how the amplitude and other properties of the echo are affected by the wing movements of flying moths.

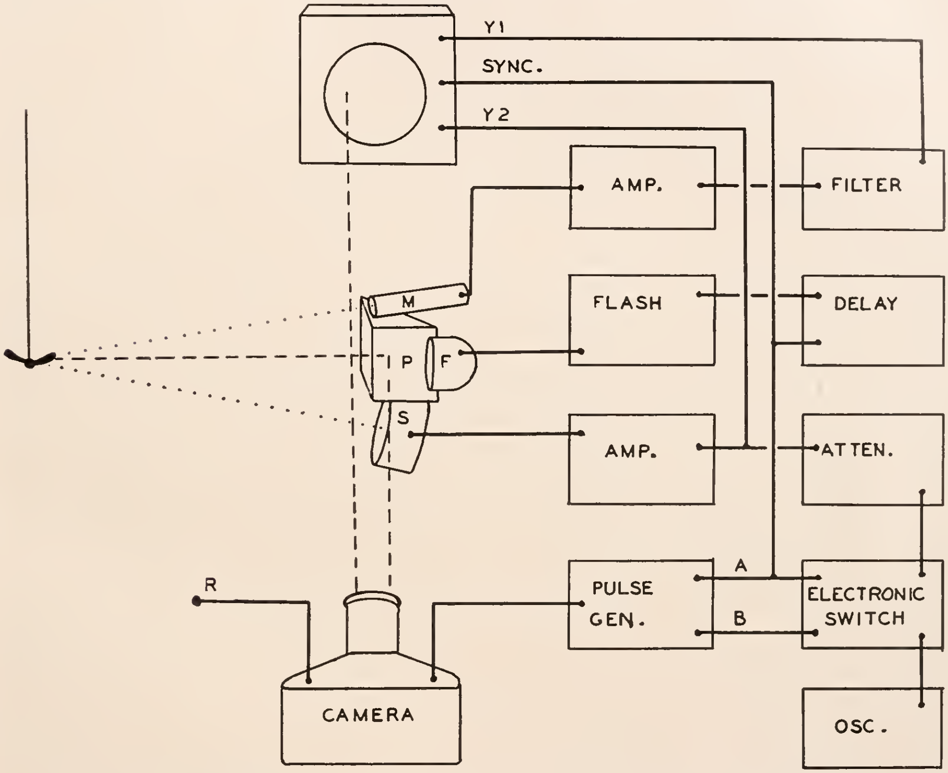

Figure 1. Block diagram of apparatus used to record the attitude and echo of a moth in fixed flight. Dotted line, acoustic path; dashed line, optical path. or other details see text.

Method

A block diagram of the apparatus is shown in Figure 1. In the early experiments a 35-mm. camera (Exakta) equipped with a 180-mm. telephoto lens framed the tube face of an oscilloscope at a distance of one meter. Half the image of the tube face was occupied by a prism (P) at 50 cm. that served to align the image of the moth next to that of the tube face. Since both images were at the same distance from the camera, the centimeter scale on the tube face (Fig. 2 *et seq.*) serves both as a time base for the echo trace and a size scale for the image of the moth.

Grouped as closely as possible around the prism were the sound source (S)—an ultrasonic transducer of the capacitative type with a membrane of 0.5 mil metallized Mylar, a Granath microphone (M) operating on the same principle, and the flash unit of a Grass PS1 Photo Stimulator (F). All three units were aimed as precisely as possible at a point to be occupied by the moth 50 cm. distant and at right angles to the camera-oscilloscope axis. The apparatus was aimed acoustically as well as visually by placing a 1-cm. sphere at this point and maximizing the echo picked up by the microphone. An attempt was made to place the axes of the microphone and transducer in relation to the optical axis so that the optical profile of the moth would correspond as closely as possible to that presented to the sound pulse.

In most of the experiments the ultrasonic pulses were between 70 and 90 kcps, 0.7 millisecond in duration, and with about 0.2 millisecond rise and fall time. Frequency modulation, of the sort encountered in the pulses of vespertilionid bats, was not employed. The ultrasonic pulses were formed by clipping the output of an oscillator (OSC.) by means of an electronic switch (General Radio). The "on" (A) and "off" (B) commands to the switch were the synchronizing and stimulus pulses from a Grass S4 stimulator. Thus, the ultrasonic pulse duration was controlled by the stimulus delay circuit of the stimulator. The flash contact in the camera triggered the stimulator each time a frame was exposed. The ultrasonic pulses thus formed passed through an attenuator and amplifier to the transmitter. In some of the experiments the signal leaving the attenuator was monitored on the lower beam (Y 2) of the oscilloscope. The sweep of the latter was triggered by the A pulse from the stimulator. A sweep speed of 1.0 millisecond/mm. was used throughout.

The A pulse also triggered the electronic flash after passing through a delay circuit. The delay was needed to insure that the image of the moth was photographed at the instant the sound pulse reached it. The picture thus reveals the moth's attitude in flight at the moment the sound pulse was reflected from its wings and body. Since the moth was approximately 50 cm. distant from the sound source the flash was delayed by 1.6 milliseconds. The duration of the flash was approximately 10 microseconds.

The microphone used to detect the echo was connected through its amplifier and a band-pass filter (to eliminate extraneous noise) to the upper beam (Y 1) of the oscilloscope.

Moths of various species were captured at light. They were mounted by cementing the mesonotum with Tackywax to an insect pin. The pin was attached to a thin vertical support of bamboo. Pin and support alone gave a negligible echo. No other object stood for a radius of several feet behind the moth, but little trouble was experienced with extraneous echoes, owing to the brevity of the pulses and the high sweep speed. The click of the camera shutter produced some acoustic interference, but this arrived later than the echo and could be disregarded.

Most moths flew spontaneously, and often for considerable periods, as soon as a paper wad in contact with the tarsi was removed. The procedure was to take a sequence of frames at random as the moth continued to fly. Sixty to 70 frames covered most of the wing positions for a given angle of presentation. Each frame was exposed for 0.01 second. The opening of the camera shutter via the cable release triggered the onset of the ultrasonic pulse, the sweep, and, after an appropriate delay, the flash. The lower trace in each frame displays the shape of the signal and the upper trace displays its echo as detected by the microphone.

Some direct interaction took place between transmitter and microphone. This occurred because a fairly intense pulse was needed in order to get an adequate echo from the moth, and because it was necessary to place transmitter and microphone as close as possible above and below the prism so as to minimize the disparity between the optical and acoustic reflections. The signal caused by this interaction appears as the first pulse on the upper trace of each recording, and serves as another control of the outgoing signal. During a given experiment it remained constant. The second signal on the upper trace, occurring about 2.5 milliseconds after the direct pulse, is the echo returned by the moth while it was in the attitude shown by the accompanying flash picture.

The still camera was replaced in later experiments by a 16-mm. motion picture camera equipped with a telephoto lens. The rotating shutter was equipped with a wiping contact that closed momentarily during the exposure of each frame. This triggered the stimulator, sound pulse, and flash as before. The camera (Ensign) was a spring-driven model, about 35 years old, in which the film speed was found to vary with the amount of pressure applied to the release button. This defect made it possible to obtain satisfactory stroboscopic pictures. With the moth in flight the camera speed was gradually increased until the number of frames per second approached the wingbeat frequency (commonly between 15 and 35 per second) of the moth. This was determined by watching the image of the moth revealed by the flashes of the strobe light. The large number of frames made available by the motion picture was of great value in the analysis.

In view of what has been said (Roeder, 1962) about the behavior of moths in the presence of ultrasound it may seem paradoxical that the moths continued to fly while being bombarded by high-intensity ultrasonic pulses. Indeed, at the beginning of a run they frequently ceased flying as soon as the sound sequence began. Some specimens were discarded as being too refractory or erratic. However, continued exposure to sound appeared to adapt the neural mechanism responsible for evasive behavior, and in most cases the moths flew steadily after a few false starts. It is fairly certain that many of the recorded wing attitudes included abrupt changes in angle of attack and amplitude associated in free moths with erratic flight. These and similar departures from "normal" flight movements are also to be expected from the restrained condition of the subjects. This problem of studying natural wing movements in insects restrained for observation has long plagued students of insect aerodynamics.

Echoes were recorded from the following species: *Sunira bicolorago* Gn., *Amphipyra pyramidoides* Gn., *Agrotis ypsilon* Rott., *Enargia decolor* Wlk., *Ennomos magnarius* Gn., *Amathes c-nigrum* L., *Graptolitha unimoda* Lintner, and *Orthosia hibisci* Gn.

Results

Figure 2 shows certain attitudes and their corresponding echoes when the axis of a flying moth is approximately at right angles to the sound path. At this angle the largest echo is produced when the wings are near the top of the stroke (A). The smallest echo is produced during the latter part of the downstroke (C), while echoes of intermediate size occur at the beginning of the downstroke (B) and near its end (D). The maximum echo produced near the top of the wing stroke is dependent upon a critical wing angle. This caused it frequently to be missed when single frames were taken. The stroboscopic motion picture method not only made a much greater number of single frames available, but it was also possible by manipulating the camera speed to hold a particular wing attitude for a number of consecutive frames. Figure 3A illustrates the contrast in magnitude between minimum and maximum echoes produced during the last half of the upstroke. In this series also the maximum echo appears to be produced when the surface presented by the wings is about 90° to the sound path. An attempt to hold the optically determined attitude of the moth constant at this point by adjusting the camera speed produced a series of attitudes that show little optical difference, although the size of the echo fluctuates widely (Fig. 3B).

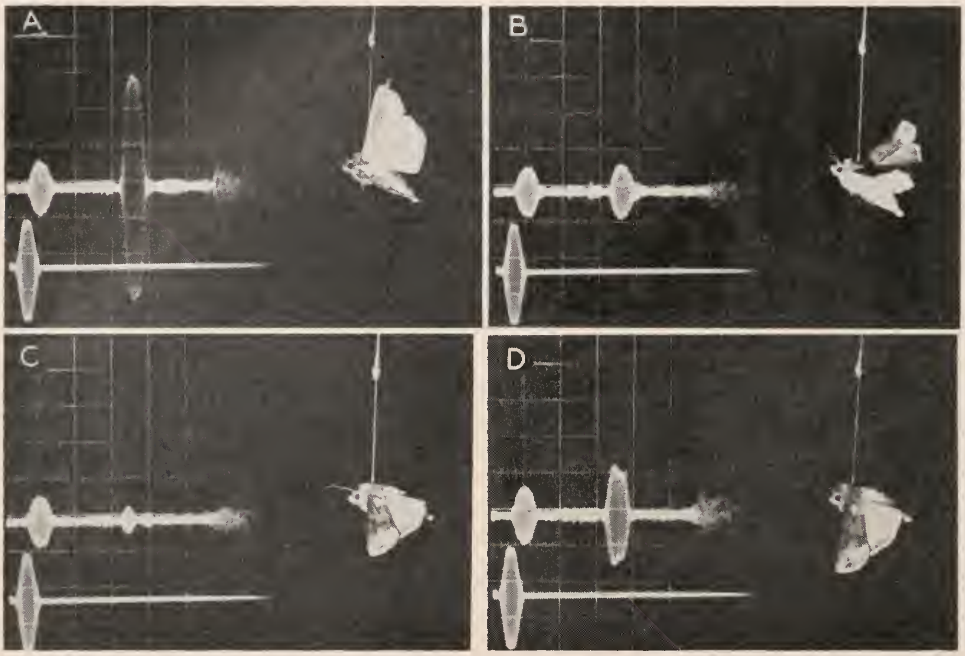

FIGURE 2. Echo and attitude of *Agrotis ypsilon* in fixed flight on bearing about 90° to sound path. Ultrasonic pulse 78 kcps, 0.7 msec. in duration. Grid on oscillogram equals 1.0 msec. on trace, 1.0 cm. on photograph. A, top of wing stroke; B, first half of downstroke; C, last half of downstroke; D, bottom of stroke. See text for other details.

From this it can be concluded that the body of the moth plays a negligible part in causing an echo, most of the acoustic reflection coming from the surface of the wings presented at 90° to the sound path. This effect may at times be further accentuated by the slightly curved surface assumed by the wings at the beginning of the downstroke. This maximum echo must occur either once or twice in rapid succession near the top of the stroke, depending upon the amplitude of the stroke and upon the precise angle of the moth relative to the sound path.

Recordings made with the axis of the potential flight path at other angles to the sound path showed similar fluctuations of echo with wingbeat, but the size of the maximum echo never reached that recorded when the axis of the

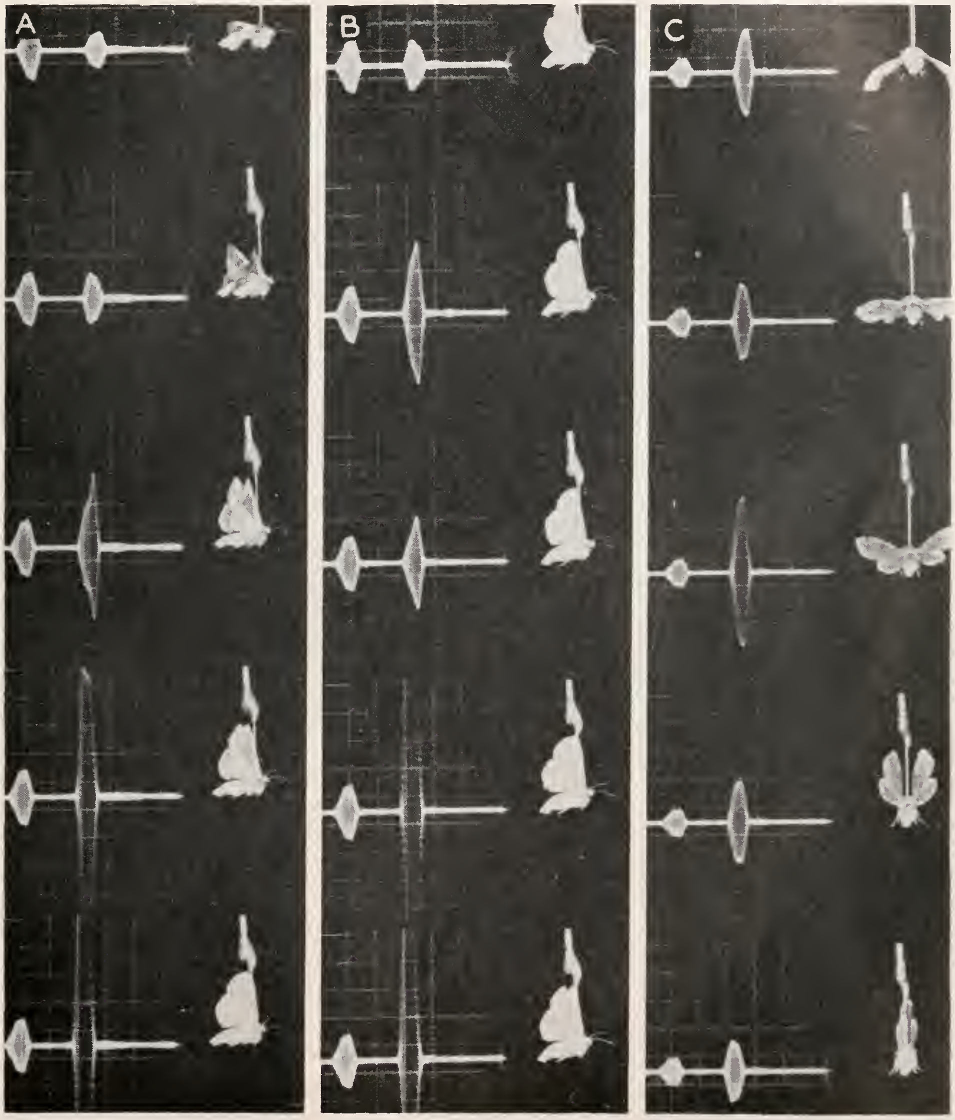

FIGURE 3. Consecutive frames from motion picture of echo and attitude of flying *Orthosia hibisci.* Ultrasonic pulse 85 kcps, 0.7 msec. in duration. Grid equals 1.0 msec. on oscillogram, 1.0 cm. on photograph. A, the second half of the upstroke. B, image held nearly stationary in stroboscopic sequence. C, path of moth directly away from sound source, wings in phases of upstroke.

moth was approximately 90° to the sound path. Samples of echoes from other angles are shown in Figure 3C, where the moth is headed directly away from the sound source, and in Figure 4, where the course is about 135°.

In the present experiments the moth was always mounted as if it were in level flight at the same altitude as the optical and acoustic system. Since the surface of the wings appears to be the main source of echoes, it is apparent from the photographs, particularly those of Figure 4, that maximal echoes would be returned from other wing positions and flight angles if the transmitter and microphone were aimed at the moth at various angles from above or below the plane of flight. These were not investigated.

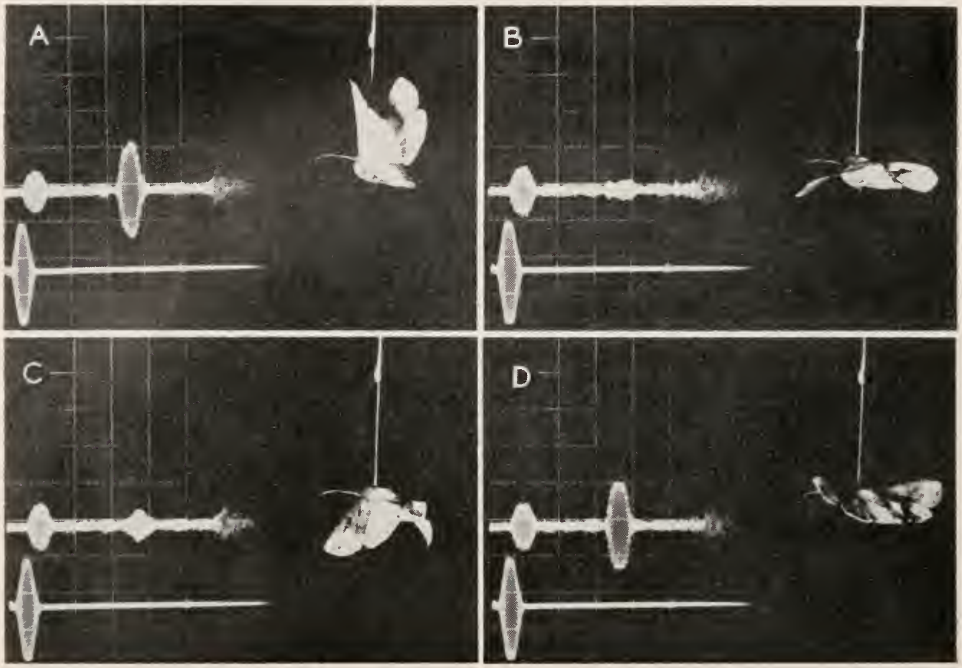

FIGURE 4. Echo and attitude of *Agrotis ypsilon* on course about 135° away from sound source. Other details as in Figure 2. A, early in downstroke. B, mid downstroke. C, early in upstroke. D, mid upstroke.

During a given run the peak echoes usually went off the oscilloscope screen (Fig. 3B) while minimum echoes sometimes disappeared in the noise level of the recording system. This made it difficult to estimate with any accuracy the intensity difference between maximum and minimum echo. In many cases it was certainly greater than 20 to 30 decibels.

Distortions of the echo were also common. The echo shown in Figure 5A has essentially the same form as the outgoing pulse. The asymmetric peak in B was probably due to movement of the wings towards the attitude producing a maximum echo during the interval of time (0.7 millisecond) that the pulse impinged upon them. The sharp peak shown in C indicates that the attitude producing a maximal echo was reached only briefly near the midpoint of the pulse. The double peaks shown in D and E may have been due to the opposite effect, the wings passing through an attitude of minimum echo during the pulse. Another possible explanation of the double peaks shown in D and E is suggested by the attitudes of the wings shown in the photographs. Echoes may have been produced separately by the near and far pair of wings. A difference in the length of the sound path from one of the two reflecting surfaces to the source by 0.5 or 1.5 wave-lengths might be expected to produce partial or complete interference and extinction of the echo. For an 82 kcps pulse of 4 mm. wavelength, such interference would occur when the wings were 1.0 or 3.0 mm. apart. This cannot be measured from the photographs, but the dimensions of the moth make it entirely possible.

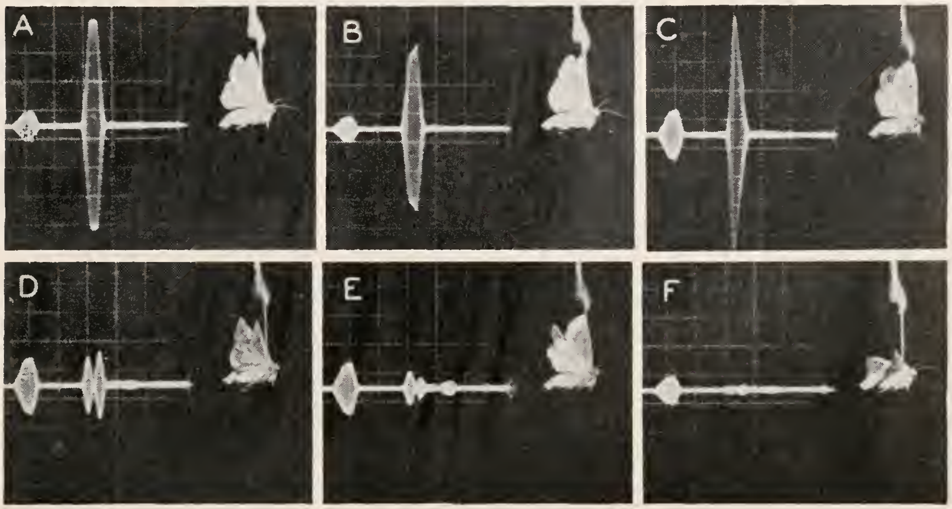

FIGURE 5. Distortions in the echo returned by *Orthosia hibisci.* Other details as in Figure 3. A, echo showing minimal distortion. B, distortion due to movement of wings to point of greater reflectance during impact of pulse. C, sharp peak caused by wings moving through position of maximum reflectance during pulse. D and E, double echoes; see text. F, abnormally small echo.

A similar explanation may account for cases where the minimum echo is below the noise level or possibly absent (Fig. 5F). Occasionally, as in D and E, a small pulse appears somewhat more than a millisecond later than the main echo. The origin of this is unknown.

Some speculation has centered on the functional significance of the scales that typically cover the wings and body of Lepidoptera. The setae covering the thorax may be extremely dense and filiform, forming a deep "fur" in many noctuids, such as *Leucania pseudargyria.* In other species, particularly in certain arctiids, this coat of scales may be very thin or almost absent, yet moths of both families and with all degrees of thoracic vestiture have well-developed tympanic organs (Haskell and Belton, 1956; Roeder and Treat, 1957) and are presumably subject to attack by bats.

A few measurements were made of the effect of this covering of scales on the echoic qualities of the subjects of the present study. Amputated wings were statically mounted so as to produce a maximum echo. They were then denuded of scales with an artist's paintbrush and the echo re-measured. Similar treatment was given to wingless bodies. Removal of the scales increased the echo by 1 or 2 decibels. Since this is an insignificant figure compared with that produced by changes in wing angles during normal flight, it was concluded that the scales play an unimportant role in reducing the echoic qualities of moths.

Discussion

From one point of view it might seem that these results demonstrate merely what could have been predicted from an elementary knowledge of the laws of wave motion. There is some comfort in this to the biologist accustomed to the unexpected in living things. The size of the echo from the wings in the attitude normal to the sound path compared with that produced by the body and the wings in other attitudes shows that the acoustic profile of a flying moth goes through much greater extremes than does its optical profile. Assuming that a bat were equipped only with a crude sonar system of the sort used in these experiments, the most definitive information that it would receive would be that its prey was flapping its wings. An optical comparison is suggested by the scintillations produced by suspended microscopic crystals, such as mica. However, in the biological situation occupied by the hypothetical bat and a flying moth, the scintillations would occur at regular intervals determined by the wingbeat frequency of the insect, and they would be maximal only when their source was travelling on certain bearings relative to the flight path of the bat.

It is certain that this postulated situation is a gross over-simplification, particularly with respect to the acoustic capabilities of the bat. Nevertheless, it does suggest some points that may have relevance in connection with the behavior of flying moths when exposed in the field to a sudden train of ultrasonic pulses (Roeder, 1962). When close to the ultrasonic source or when exposed to pulses of high intensity many moths react by changing from a relatively straight flight path to a variety of turns, spirals, and dives. Others close their wings and fall passively to the ground. The experiments reported here suggest that cessation of flight movements and closure of the wings must eliminate the major source of echoes, as well as the echo fluctuation characteristic of flight, thereby providing the insect with some measure of acoustic concealment as it falls to the ground.

In the same paper it was reported that moths flying at greater distances from the sound source and exposed to lower intensities frequently turned from their flight path and flew directly away from the sound source. This maneuver has the obvious advantage to the moth in putting distance between it and its potential predator, but the echo experiments suggest that it may have additional survival value. Moths flying at roughly the same altitude as an approaching bat are most likely to present an optimum target for echoes if they cross the flight path of the bat at about 90° (see Fig. 3). The first clue available to a bat approaching at maximum range must be a very brief echo of its cry occurring once or twice for each wingbeat of the target. Since at least some noctuid moths are certainly capable of detecting the echolocating cries of a bat at a considerably greater range than a bat can detect their echoes (Roeder and Treat, 1961), it must be of some advantage to the moth to assume a less echo-producing flight path, *e.g.,* parallel to or directly away from that of the bat, as soon as the latter has been detected.

The situation is much more difficult to assess from the viewpoint of the bat, for its frequency-modulated cry is more complex than the pulses used in these experiments and little is known about its capabilities of acoustic discrimination. Relatively long pulses, such as the cruising pulses emitted by *Myotis lucifugus* (Griffin, 1958), might increase the bat's chances of picking up a brief maximal echo from a flying moth. For instance, a cry 15 milliseconds in duration would last throughout one half of the wingbeat, *i.e.,* for the whole upstroke or downstroke, of a moth flapping its wings 30 times a second. The echo returning to the bat would be amplitude-modulated with a sharp peak at one point. At extreme range the brief peak would be the only part of the echo detected by the bat.

Detection of an echo causes most bats to increase the repetition rate of their cries to as much as 150 per second. At the same time there is a decrease in the duration (to 1.0 millisecond or less) and in the frequency (to about 25 kcps) of each pulse of sound. Most noctuid moths have wingbeat frequencies of between 10 and 40 per second. Therefore, when reception of an echo causes the bat to increase its pulse repetition rate a point must be reached where there is phasic interaction between pulse frequency and echo frequency determined by the moth's wingbeat. At some frequencies the phasing of pulse and echo source would produce a maximal echo every time, while at others the echo would be missed entirely. The signal significance of this effect, as well as the role played by frequency modulation in the bat's cry, cannot be estimated at present.

The experimental work was supported by Grant E-947 from the United States Public Health Service to Tufts University.

Some of the equipment was loaned by Professor Donald R. Griffin of Harvard University. The moths used were identified by Dr. Asher E. Treat of the City College of New York.

SUMMARY

1. Moths of several species were mounted in stationary flight and the echoes of ultrasonic pulses were recorded simultaneously with flash photographs of the attitude assumed by the wings at the instant the pulse reached the insect.

2. The largest echo was produced by moths flying at the same altitude as the sound source when the potential course was roughly at right angles to the sound path and the wings were near to the top of the stroke. The difference between this maximum echo and that produced by the body and wings at other attitudes of the wing stroke was 30 decibels or more.

3. Moths flying at the same altitude as the sound source but on other courses produced echoes that fluctuated with wing position. However, the maximum was never as great as that registered on the 90° course.

4. Distortions in the shape of the echo are described and their causes are discussed. Scales on the wings or body of the moth do not appear to play an important anechoic role.

5. It is concluded that the plane surface of the wings returns the major portion of the echo. The significance of this is discussed in relation to the problems of detection and evasion encountered under natural conditions by bats and flying moths.

LITERATURE CITED

Griffin, Donald R., 1958. Listening in the Dark. Yale University Press, New Haven, Conn.

Griffin, Donald R., Frederic A. Webster and Charles R. Michel, 1960. The echolocation of flying insects by bats. *Animal Behaviour,* **8**: 141–154.

Haskell, P. T., and P. Belton, 1956. Electrical responses in certain lepidopterous tympanic organs. *Nature,* **177**: 139–140

Kolb, A., 1958. Über die Nahrungsaufnahme einheimischer Fledermäuse vom Boden. *Verh. Deutsch. Zool. Gesellsch. im Frankfurt a.M.,* 162–168.

Möhres, F. P., 1950. Aus dem Leben unserer Fledermäuse. *Kosmos,* **46**: 291–295.

Roeder, Kenneth D., 1962. The behaviour of free flying moths in the presence of artificial ultrasonic pulses. *Animal Behaviour,* **10**: 300–304.

Roeder, Kenneth D., and Asher E. Treat, 1957. Ultrasonic reception by the tympanic organ of noctuid moths. *J. Exp. Zool.,* **134**: 127–158.

Roeder, Kenneth D., and Asher E. Treat, 1961. The detection and evasion of bats by moths. *Amer. Sci.,* **49**: 135–148. (Reprinted in 1961 Annual Report of Smithsonian Institution.)

Treat, Asher E., 1955. The response to sound in certain Lepidoptera. *Ann. Ent. Soc. Amer.,* **48**: 272–284.

Webster, Frederic A., 1963. Bat-type signals and some implications. Human Factors in Technology (eds., E. Bennett, J. Degan, and J. Spiegel). McGraw-Hill Book Co., Inc., New York, N. Y.

Webster, Frederic A., and Donald R. Griffin, 1962. The role of the flight membranes in the capture of insects by bats. *Animal Behaviour,* **10**: 332–340.

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ENZYMIC HISTOCHEMISTRY OF GRANULAR COMPONENTS IN DIGESTIVE GLAND CELLS OF THE ROMAN SNAIL, HELIX POMATIA[1]

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The digestive gland of *Helix pomatia* has been studied histologically especially during feeding and digestion (Krijgsman, 1925, 1929; Thiele, 1953; Guardabassi and Ferreri, 1953 among others). In addition, biochemical studies employing homogenates of gland tissue or aliquots of crop or digestive fluid have established that both intra- and extracellular hydrolytic enzymes are associated with the gland tissue (Holden and Tracey, 1950). Such methods, while providing quantitative data, do not permit direct study of the intracellular enzymic activity within the gland. *Helix* appears able to produce enormous amounts of several hydrolytic enzymes rapidly (Holden and Tracey, 1950; Billett, 1954; Dodgson and Powell, 1959) and it is therefore not unreasonable to assume that a highly developed synthetic machinery for secretion of extracellular hydrolases may exist within the cells concerned.

The activity of several hydrolytic enzymes, including acid phosphatase, β-glucuronidase, and several "cathepsins," has been shown by biochemical methods to vary according to the feeding cycle (Holden and Tracey, 1950; Jarrige and Henry, 1952). Several histochemical studies have shown β-glucuronidase activity (Billett and McGee-Russell, 1955) and acid and alkaline phosphatase activity (Guardabassi and Ferreri, 1953; Nakazima, 1956) within digestive gland tubules. To date, no reports have dealt with visualization of enzymic activity within specific digestive gland cells.

In this investigation we were concerned not only with achieving intracellular localization of specific hydrolases, but also with a comparison of intracellular enzymic activity during periods of starvation and active feeding. We were especially interested in the identification of enzymic activity with specific intracellular granules. The position of these granules has been shown to vary during feeding and digestion (Krijgsman, 1929; Rosen, 1941). With cytochemical methods, we were hopeful that it would be possible to extend these earlier cytological observations and establish a more precise role for these granules consistent with some recent concepts of intracellular digestion and related hydrolytic enzymic activity.

MATERIALS AND METHODS

Our initial stock culture consisted of mature, estivating specimens of *Helix pomatia* from Morocco.[2] The snails were activated by exposure to a warm, humid environment. Animals were deemed "fed" after they had been observed to feed continuously on fresh lettuce leaves for several hours following starvation for 5–7 days. Animals were considered "starved" when they had been isolated in individual fingerbowls for at least 7 days without food, or when they were killed while in estivation. For some experiments, animals were fed for at least 24 hours on fresh lettuce leaves soaked in a solution (25 mg. per 1 ml. water) of horse-radish peroxidase.

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[1] This study was supported by grants from the United States Public Health Service (RG-5483 and A-3605) and a contract with the Office of Naval Research.

[2] Courtesy of Miscuraca Importing Corp., New York, N. Y.

The digestive gland was located by removing the apex of the shell and uncoiling the animal, the gland being identified as a green-brown mass near the upper end of the coil. For histochemical purposes, tissue was cut into small pieces (1–2 mm.) immediately following removal and fixed in cold (4° C.) calcium-formalin (Baker's) or cold chloral hydrate formalin (Fishman and Baker, 1956) for 18–24 hours. Other pieces of gland tissue were fixed in aqueous Bouin's fluid, alcohol-formol-acetic acid fixative or Carnoy's fluid.

Prior to being sectioned on a freezing microtome, digestive gland tissue must be embedded in gelatin, owing to its friable nature. Fixed tissue was washed briefly in cold water and placed for no longer than one hour in 15% gelatin at 37° C. and then hardened at 0° C. for 20 minutes, and by additional treatment in cold 10% neutralized formalin for one hour. Immediately prior to sectioning, the block was briefly rinsed in cold water.

Enzymes: Acid phosphatase activity was visualized by the lead-salt method of Gomori (1952), with β-glycerophosphate as substrate, and by the azo dye method of Burstone (1958) with naphthol AS-MX phosphate as substrate and Red Violet LB as coupling reagent following cold acetone treatment (20 minutes) to remove lipid. With both methods, sections were incubated for 20–60 minutes at 37° C. (Rosenbaum and Rolon, 1962). For visualization of *β-glucuronidase* activity, we employed cold chloral-hydrate-formalin-fixed tissue with 8-hydroxyquinoline glucuronide as substrate (Fishman and Baker, 1956). Sections were incubated at 37° C. for 30 minutes to 6 hours. For *non-specific esterase* activity, the pararosanilin method of Lehrer and Ornstein (1959) was used following exposure of fixed sections to cold acetone. Incubation proceeded at 0° C. for 30 minutes to two hours with α-naphthyl acetate as substrate. A parallel series of tissues was exposed to the organophosphorous compound E-600 (diethyl-*p*-nitrophenyl phosphate, 10^{-5} M in Tris-maleate buffer, pH 7.2) for one hour at 37° C. prior to incubation. This procedure has been considered capable of demonstrating type-C esterases (Hess and Pearse, 1958). For localization of *aminopeptidase* activity, we employed the method of Burstone and Folk (1956), using the substrate L-leucyl β-naphthylamide at pH 7.1 (0.2 M Tris buffer) following treatment of sections in cold acetone. Simultaneous coupling was obtained with the diazonium salt, Garnet GBC.

Other histochemical methods: Detection of phospholipid was performed with the acid hematein method of Baker (1946). Some paraffin-embedded tissues fixed in Bouin's and Carnoy's fixatives were stained by the periodic acid Schiff method following digestion with salivary amylase. Finally, visualization of exogenous peroxidase, employed as a "marker" in some experiments, was accomplished by use of a hydrogen peroxide substrate and benzidine, essentially as described by Straus (1959).

RESULTS

Cytology of the digestive gland: Several authors have presented good descriptions of the kinds of cells in the digestive gland of *Helix* (Krijgsman, 1929; Rosen, 1941; Thiele, 1953). This work has resulted in a variety of terms frequently descriptive of the same cell type. It is desirable, therefore, to clarify our nomenclature for the cell types considered in the present study.

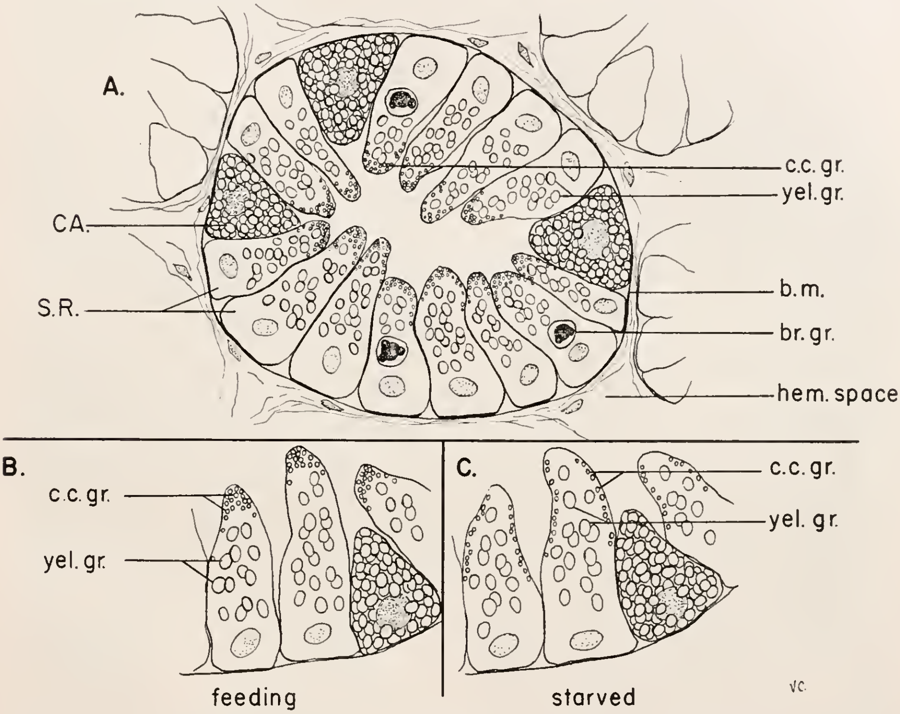
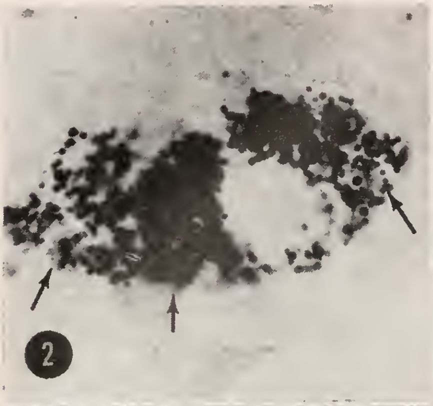
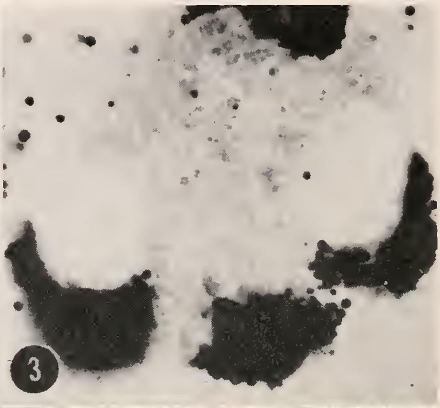
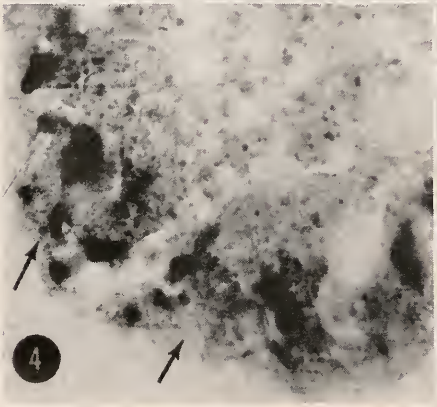


FIGURE 1. A. Schematic drawing of a digestive gland tubule from *Helix pomatia,* showing relationships of the different cytological components. B. Distribution of various intracellular granules during the feeding stages of *Helix.* Clear colorless granules are almost exclusively localized at the luminal border of the secretory-resorption cells. C. In secretory-resorption cells from starved animals, the clear, colorless granules are not concentrated at the luminal border. CA, calcareous cell; SR, secretory-resorption cell; c.c., gr., clear, colorless granules; yel. gr., yellow granules; b.m., basement membrane; hem. space, hemocoelic space.

Our observations concern two kinds of cells (Fig. 1)—the so-called *calcium cell* and the *digestive* or *SR cell* (secretory-resorption cell—Rosen, 1941). The *calcium cell* is characterized by its triangular shape with the broad base (approximately 50 μ) touching the basement membrane. The cell points toward, but does not extend into, the tubular lumen. The cytoplasm contains numbers of large, nearly colorless spherules, the so-called *calcium spherules. Secretory-resorption* (SR) *cells* are greater in number than, and adjacent to, the calcium cells. The SR cells are long (100 μ), the body of the cell extending from the basement membrane into the lumen of the glandular tubule. The nucleus is generally smaller than that of the calcium cell. The SR cell possesses granular inclusions, the number and distribution of which vary considerably with the feeding stage of the animal (Krijgsman, 1929). These granules are generally described according to three types: *large brown granules* not present in all cells, but, when present, con-







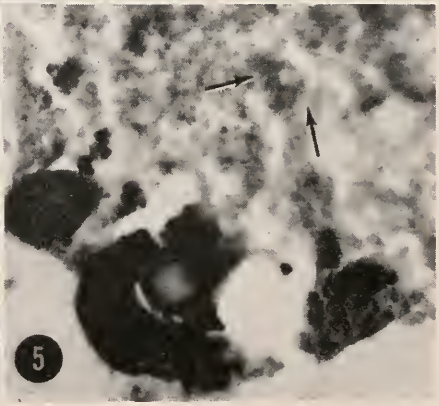


Figure 2. Three calcium cells (arrows) from the digestive gland of a starved snail, stained with the method of Gomori, omitting glyercophosphate from the incubation medium. Calcium spherules are stained by lead sulfide. All other regions of the digestive gland are negative. 360 ×.

Figure 3. Four calcium cells from the digestive gland of a recently fed snail, stained as in Figure 2 but with glycerophosphate present as substrate. Calcium spherules, stained by the false positive reaction, are obscured by an intense cytoplasmic staining due to enzymic activity. A few spherules have been scattered extracellularly in processing. 360 ×.

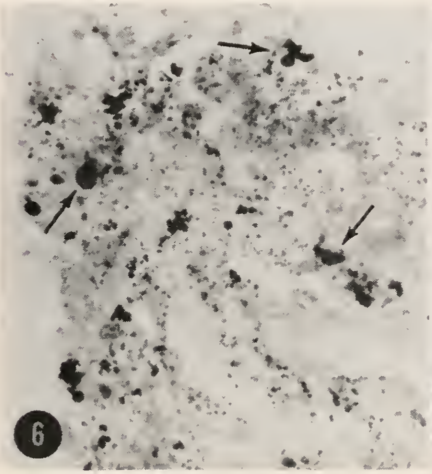
Figure 4. Tubule from the digestive gland of a starved animal, stained for acid phosphatase activity by the azo dye method of Burstone. Enzymic activity is present in calcium cells (arrows), and weaker activity is present in the cytoplasm of SR cells. Spherules do not stain. Green filter. 360 ×.

Figure 5. Tubule from the digestive gland of a recently fed animal, stained as in Figure 4. Intense enzyme activity is seen in the cytoplasm of three calcium cells. The spherules do not stain. The cytoplasm of the SR cells is heavily stained, especially near the lumen (arrows). Green filter, 360 ×.

tained within vacuoles located at the base of the cell in the vicinity of the nucleus; *small yellow granules* generally distributed throughout the cytoplasm; *small clear granules* apparently free in the cytoplasm near the periphery of the cell. The distribution of all granule types is summarized in Figure 1A.

Enzyme distribution within the gland cells of starved and feeding animals

Acid phosphatase: With the Gomori metal-salt method, we could not distinguish acid phosphatase activity in the cytoplasm of calcareous cells since calcium spherules from both starved and feeding animals blackened intensely with the sulfide. These calcium granules were large, round bodies, which frequently became dispersed outside the cell due to compression of the sections during processing (Figs. 2 and 3). Application of heat to the sections (90° C. for at least 10 minutes), or omission of glycerophosphate substrate from the incubation medium, demonstrated that this localization of final reaction product was a false positive reaction not due to enzymic activity. With use of an azo dye method for acid phosphatase activity, the spherules showed no enzymic activity (Figs. 4 and 5). With staining by the azo dye method, however, it was clear that the cytoplasm of calcareous cells from recently fed animals showed increased acid phosphatase activity over cells from starved animals (compare Figs. 4 and 5).



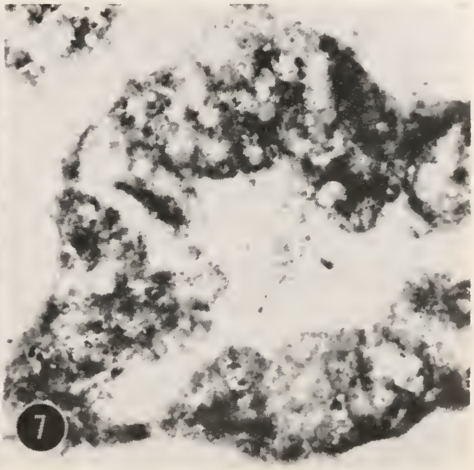
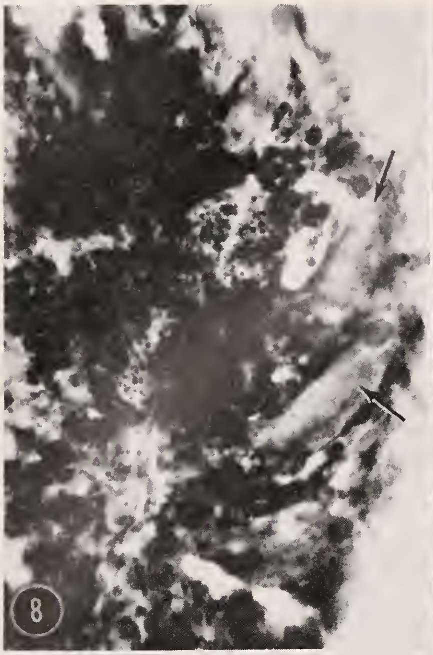
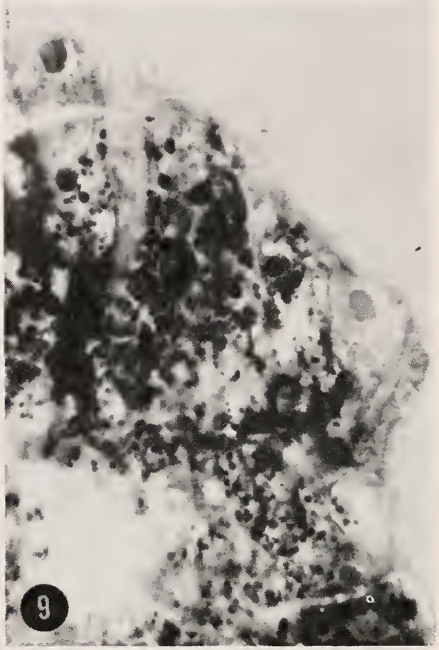


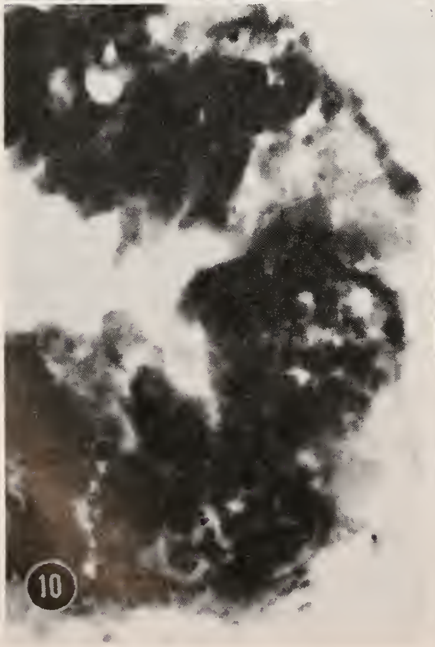
FIGURE 6. Digestive gland tubules from a starved animal, stained for β-glucuronidase activity by the method of Fishman and Baker, following fixation in cold chloral-hydrate-formalin. Enzymic activity is confined to the yellow granules. Brown granules (arrows) did not stain for enzymic activity but are emphasized by the yellow filter employed. Calcium cells are negative. 200 ×.

FIGURE 7. Digestive gland tubule from a recently fed animal, stained as in Figure 6. There is increased cytoplasmic activity, especially at the periphery of SR cells. The yellow granules are also stained. Calcium cells are negative. Yellow filter. 200 ×.

The SR cells from starved animals showed little or no enzymic activity by either the azo dye or lead-salt methods. In fed animals, some enzymic activity could be detected near the luminal border in the region where the small, colorless







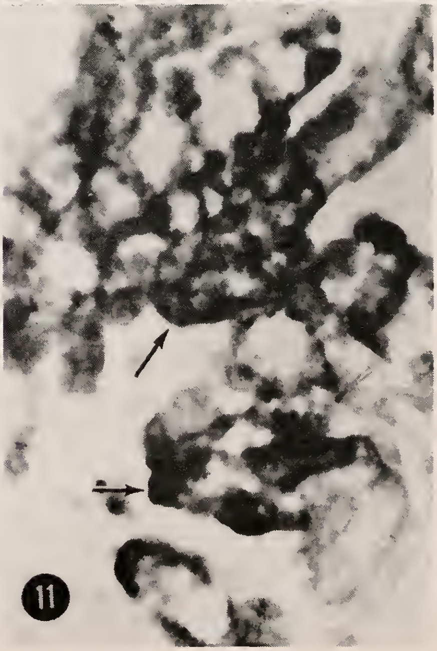


FIGURE 8. Digestive gland tubule from a starved animal, stained for non-specific esterase activity by the method of Lehrer and Ornstein. There is intense cytoplasmic staining, and staining of granules is evident in SR cells. Calcium cells (arrows) are negative. Green filter, 380 ×.

granules accumulated. Neither the small yellow nor large brown granules possessed demonstrable acid phosphatase activity by the methods we employed.

When sections were exposed to the non-ionic surface-activating agent, Triton X-100 (0.25% at 4° C. for one hour), a distinct decrease of enzymic activity within calcium cells could be detected with the azo dye method, especially in tubules from starved animals. With identical treatment applied to tissue from feeding animals, enzymic activity in calcium cells and at the lumen of SR cells was also diminished.

β-Glucuronidase (*β-glucosiduronidase*): Calcium cells showed no activity for this enzyme. In SR cells from starved animals, intracellular enzymic activity could be detected in locations approximating that of the yellow granules (Fig. 6). The large brown granules showed no enzymic activity. Diffuse staining of the peripheral cytoplasm occurred in locations consistent with those of the small colorless granules. In secretory-resorption cells from fed animals, staining for β-glucuronidase activity was intense and generally distributed throughout the cytoplasm. Activity for this enzyme also appeared in the yellow granules (Fig. 7).

Esterases: Calcium cells from both starved and feeding animals showed no esterase activity (Figs. 8 and 9). Secretory-resorption cells stained intensely for non-specific esterase activity. Activity appeared throughout the cytoplasm (Fig. 8), but with fed animals the reaction was more intense and diffuse (Fig. 10) than with starved animals. In all cases, staining for non-specific esterase activity could not be identified with specific granules.

In both starved and feeding animals, treatment with the organo-phosphorous inhibitor E-600 resulted in a considerable loss of cytoplasmic activity. In preparations from starved animals, E-600-resistant esterase activity was limited to what appeared to be the yellow granules of the SR cells (Fig. 9). Treatment of sections from fed animals in E-600 resulted in a diffuse localization of enzymic activity within peripheral regions of SR cells (Fig. 11). This region generally contained the small, colorless granules.

Aminopeptidase: Calcium cells from both starved and feeding animals showed no enzymic activity. Some slight activity was detected in secretory-resorption cells from starved and feeding animals.

Other histochemical methods

Phospholipid: With Baker's acid hematein method for phospholipid, positive staining occurred in the peripheral region of SR cells from starved and feeding animals (Figs. 12 and 13). Localization of phospholipid in SR cells in the glands of starved animals was more diffuse (Fig. 12), while in fed animals it was limited

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FIGURE 9. Digestive gland tubule from a starved animal, stained as in Figure 8, following treatment of the section in the organo-phosphorous inhibitor E-600. Non-specific enzyme activity present in the cytoplasm of cells in Figure 8 has been removed but enzymic activity remains in yellow granules scattered throughout the SR cells. Calcium cells are negative. Green filter. 380 ×.

FIGURE 10. Digestive gland tubule from a feeding animal, stained for non-specific esterase activity as in Figure 8. There is intense enzyme activity in SR cells. Green filter. 380 ×.

FIGURE 11. Digestive gland tubule from a recently fed animal. The section was treated with E-600 and stained for esterase activity as in Figure 9. While general cytoplasmic enzyme activity is diminished, there is positive staining for E-600-resistant enzyme activity near the lumen of the tubule (arrows). The calcium cells are negative. Green filter. 380 ×.

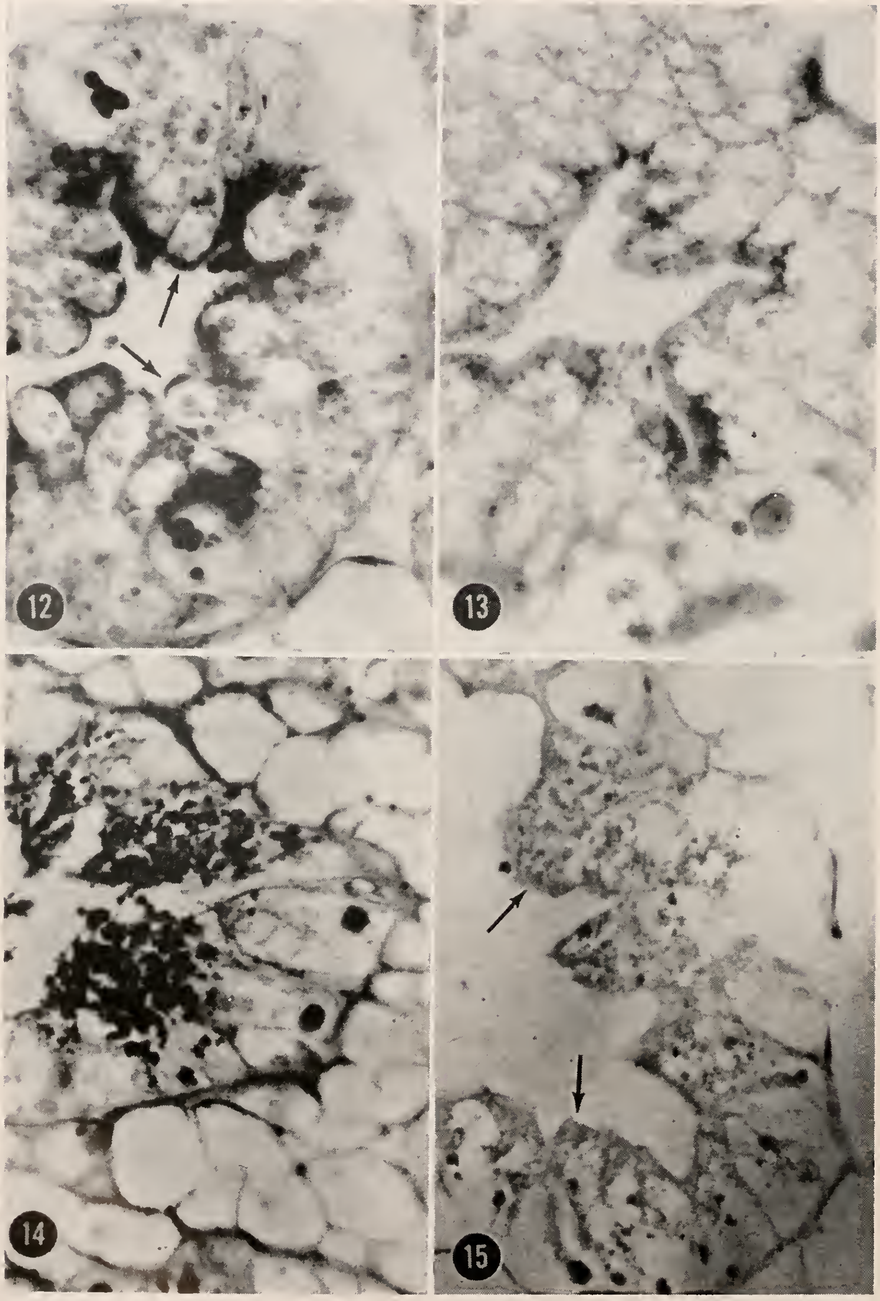

Figure 12. Tubule from the digestive gland of a starved animal, stained for phospholipid by the method of Baker. There is peripheral staining of SR cells, both at the luminal border (arrows) and the lateral cell margins. Brown granules, although detectable, are not stained with this method. 360 ×.

to the luminal border (Fig. 13). The location of phospholipid staining corresponded to areas containing the small, colorless granules. A weak reaction for phospholipid was present throughout the cytoplasm of SR cells in both starved and feeding animals. There was no staining of the calcium cells.

PAS reaction: The periodic acid Schiff reaction was employed following incubation with amylase at pH 7.4 for digestion of glycogen. In secretory-resorption cells from starved animals, the yellow granules were intensely Schiff-positive (Fig. 14). In cells from fed animals, fewer yellow granules were present and these showed a diminished Schiff reaction (Fig. 15). In glands from fed animals fixed in aqueous Bouin's fluid, a weak Schiff-positive reaction appeared at the luminal border of the secretory-resorption cells (Fig. 15). This region corresponded to where localized phospholipid was detected and where, in fed animals, the small colorless granules were always observed.

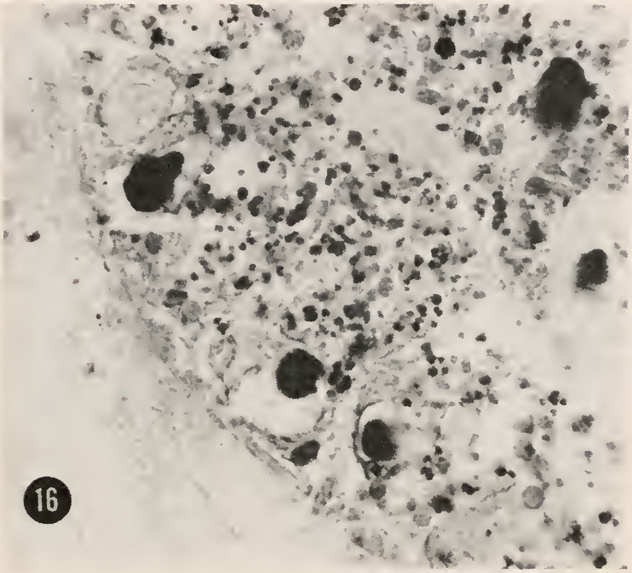

FIGURE 16. Digestive gland tubule from a snail fed on lettuce soaked in horse-radish peroxidase, visualized by means of a benzidine reaction. Numerous "vacuoles" with enzyme activity appear throughout SR cells. Brown granules are also stained by the benzidine reaction but do not possess enzyme activity. Yellow filter. 360 ×.

Ingestion of peroxidase: Within 15 minutes following initial attachment of a snail onto a leaf of treated lettuce, peroxidase could be detected in the cytoplasm

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FIGURE 13. Tubule from the digestive gland of a fed animal stained for phospholipid as in Figure 12. The reaction is present chiefly at the luminal border of SR cells. 360 ×.

FIGURE 14. Tubule from the digestive gland of a starved snail, fixed in Bouin's fluid and stained with periodic acid Schiff method following digestion with saliva. The yellow granules within SR cells appear intensely Schiff-positive. Green filter. 360 ×.

FIGURE 15. Digestive gland tubule from a fed animal, stained as in Figure 14. A few yellow granules are present but weakly stained. There is staining of the luminal border of SR cells, corresponding to the location of clear, colorless granules (arrows). Green filter, 360 ×.

of the secretory-resorption cells (Fig. 16). Localization of ingested enzyme appeared to be within "vacuoles" scattered throughout the cytoplasm, especially near the luminal border of the SR cells. The location of the larger peroxidase-positive "vacuoles" corresponded to that of the yellow granules. With extended feedings (up to three hours) on treated lettuce, we could detect no increase in the amount of peroxidase ingested, nor did existing sites of peroxidase activity coalesce to form larger vacuoles.

Control experiments on cells from animals fed untreated lettuce revealed no peroxidase activity endogenous to the snail itself.

Discussion

In this study, hydrolase activity was localized within secretory-resorption cells and calcium cells from the digestive gland of *Helix*. The location of some of this enzymic activity closely corresponded to the distribution of migrating intracellular granules described by Krijgsman (1925, 1929) and by Rosen (1941).

The activities of the hydrolases considered here have previously been visualized primarily in cells from vertebrate tissues, where their role in resorption phenomena or intracellular digestion has been described. Thus, acid phosphatase activity has been identified within phagocytic macrophages (Weiss and Fawcett, 1953), with iron resorption in liver (Novikoff and Essner, 1960) and protein resorption in kidney (Straus, 1961). The activity of two other hydrolases has been visualized in the peribiliary region of vertebrate liver cells (esterase—Holt, 1956; β-glucuronidase—Goldfarb and Barka, 1960), where their role in resorption was suggested. Studies with invertebrates have identified intracellular hydrolytic activity with feeding and digestion in planarians (Rosenbaum and Rolon, 1960), amebas (Novikoff, 1959; Birns, 1960) and ciliate protozoa (Seaman, 1961; Rosenbaum and Wittner, 1962).

Unfortunately, those few studies describing the activity of hydrolytic enzymes in molluscan digestive glands (see review of Arvy, 1962) employed methods no longer deemed completely reliable for purposes of intracellular localization. In the present investigation, we attempted to increase reliability of the methods by minimizing enzyme diffusion and inactivation through use of frozen sections of tissue fixed briefly in the cold. Such methods, especially in combination with newer naphthol substrates and rapid simultaneous coupling to a diazonium salt, offered additional protection against diffusion of enzyme, final colored reaction product, or both (Rosenbaum, 1962). Control of these factors served to support our observations that much, if not all, of the intracellular enzymic activity we studied was associated with granular or with vacuolar structures within the cells concerned.

Within a short time following ingestion of food, the secretory-resorption cells showed increased activity for several enzymes, especially β-glucuronidase and esterases. Much of this activity was associated with yellow granules, although nonspecific esterase activity was also diffusely distributed in the cytoplasm of secretory-resorption cells. It is noteworthy that the yellow granules also served as sites of exogenous horse-radish peroxidase accumulation. We propose that this enzyme entered the cell by pinocytosis, perhaps via the clear, colorless granules, which may represent small pinocytic vacuoles. The observation that these small granules stained for phospholipid and were also Schiff-positive suggests a possible relationship to the cell membrane. During feeding, the region of accumulation of these clear, colorless granules also possessed increased E-600-resistant esterase activity.

The present observations did not permit determination as to whether the yellow granules in the secretory-resorption cells could form vacuolar structures associated with the granules as resorption took place. The accumulation of pinocytic vacuoles associated with migrating granular structures has been described in specialized vertebrate cells grown in tissue culture (Rose, 1957a, 1957b). In *Paramecium*, Rosenbaum and Wittner (1962) have described migration of neutral red-staining granules from a region beneath the pellicle toward forming food vacuoles as these become larger and begin to show increased activity for acid phosphatase, non-specific and E-600-resistant esterases during feeding. In addition to this enzymic activity, the neutral red bodies of the paramecium stained for phospholipid, and their possible relationship to vertebrate hepatic lysosomes (deDuve, 1959) was therefore suggested. We have made no observations with respect to the ability of granular components within the SR cells of *Helix* to stain selectively with neutral red.

The present study points also to the possibility that intracellular digestion may take place within secretory-resorption cells of the digestive gland. Phagocytosis, which is closely related to pinocytosis, occurs in *Helix* (Krijgsman, 1929; Rosen, 1941; van Weel, 1961), although its role in feeding or digestion has been little studied. However, a pinocytic mechanism, permitting active absorption of dissolved food substances, would be a more attractive one than simple "diffusion," the process suggested by earlier investigators (Hirsch, 1915; Jordan and Begemann, 1921). There is no question that the first stage of digestion in the snail depends on a large number of enzymes acting in the gut lumen (Holden and Tracey, 1950; van Weel, 1961). Such extracellular enzymic activity could serve to break down ingested food initially, thereby permitting further intracellular digestion to take place.

Our present study does not test the possibility that migration of the clear colorless granules to the luminal border of the secretory-resorption cells during feeding could be related to discharge of enzymes into the lumen. The presence of increased E-600-resistant esterase activity at the luminal border during feeding might represent a stage in the release of proteolytic enzymes into the glandular lumen. It would seem, however, that initially extracellular digestion in the gut of an herbivorous species such as *Helix* would be brought about primarily by carbohydrases, not by enzymes more specifically suited to protein hydrolysis. It has been suggested (van Weel, 1961) that the salivary glands chiefly produce carbohydrases, and even that some of these enzymes are not produced by the animal itself (Florkin and Lozet, 1949; Jeuniaux, 1954). Once food has reached the gut and digestion begun, however, succeeding steps must involve some degree of intracellular digestion, especially of protein. Activity of the intracellular hydrolases described in the present study would appear to be well suited for intracellular breakdown of plant protein.

Summary and Conclusions

1. Cytochemical visualization methods for activity of acid phosphatase, β-glucuronidase, aminopeptidase and non-specific and E-600-resistant esterases were

applied to digestive gland tissue from starved and feeding *Helix pomatia*. Other cytochemical methods used included Baker's acid hematein for phospholipid and the periodic acid Schiff method.

2. Calcareous cells stained only for acid phosphatase activity, by both lead-salt and an azo dye method. Calcium granules within these cells did not stain with the azo dye method while false-positive reactions in the granules were always obtained with the lead-salt method. Some increase in enzymic activity was detected in the cytoplasm of feeding animals. Secretory-resorption (SR) cells showed little activity for acid phosphatase.

3. The yellow granules in SR cells stained for β-glucuronidase activity in both starved and feeding animals. After feeding, SR cells showed an increase in enzymic activity, both in granules and more diffusely in the cytoplasm. Although some aminopeptidase activity was present, insignificant differences in location and intensity of the enzyme were detected.

4. SR cells stained intensely for non-specific esterase activity. Cytoplasmic staining for this class of enzymes was intense in feeding animals. Treatment with the inhibitor E-600 resulted in loss of cytoplasmic staining; activity persisted in yellow granules in both starved and feeding animals. Some activity was detected in small, colorless granules.

5. Phospholipid was detected in peripheral regions of the SR cells from starved and feeding animals. The reaction was diffuse in starved animals but concentrated at the luminal border of the SR cells in fed animals. Yellow granules of SR cells were periodic acid Schiff-positive. Especially in fed animals, periodic acid Schiff granules appeared at the luminal border of SR cells. Starved animals fed on lettuce leaves impregnated with horse-radish peroxidase showed accumulation of enzyme in vacuoles closely associated with the yellow granules.

6. The observations extend the concepts, advanced by earlier workers, that granular components of the secretory-resorption cells play a significant role in digestion in the snail. The location of activity of the several hydrolases studied, and the alterations in response to feeding, suggest that these classes of enzymes and the granules form a functioning unit in the physiology of intracellular digestion in *Helix*.

LITERATURE CITED

Arvy, L., 1962. Histochemie des enzymes impliqués dans la digestion, dans la série animale. *Handbk. Histochem.* VII, 2: 154–303.

Baker, J. R., 1946. Histochemical recognition of lipine. *Quart. J. Micr. Sci.*, **87**: 441–470.

Billet, F., 1954. The β-glucuronidase of the Roman snail (*Helix pomatia*). *Biochem. J.*, **57**: 159–162.

Billet, F., and S. M. McGee-Russell, 1955. The histochemical localization of β-glucuronidase in the digestive gland of the Roman snail (*Helix pomatia*). *Quart. J. Micr. Sci.*, **96**: 35–48.

Birns, M., 1960. The localization of acid phosphatase activity in the amoeba, *Chaos chaos*. *Exp. Cell Res.*, **20**: 202–205.

Burstone, M. S., 1958. Histochemical demonstration of acid phosphatase with naphthol-AS phosphates. *J. Nat. Cancer Inst.*, **21**: 523–540.

Burstone, M. S., and J. E. Folk, 1956. Histochemical identification of aminopeptidase. *J. Histochem. Cytochem.*, **4**: 217–226.

Dodgson, K. S., and G. M. Powell, 1959. Studies on sulphatases. 26. Arylsulphatase activity in the digestive juice and digestive gland of *Helix pomatia*. *Biochem. J.*, **73**: 666–671.

deDuve, C., 1959. Lysosomes, a new group of subcellular particles. In "Subcellular Particles" (ed. by T. Hayashi), pp. 128–158. Ronald Press, New York.

Fishman, W. H., and J. R. Baker, II, 1956. Cellular localization of β-glucuronidase in rat tissue. *J. Histochem. Cytochem.,* **4**: 570–587.

Florkin, M., and F. Lozet, 1949. Origine bactérienne de la cellulase du contenu intestinal de l'escargot. *Arch. int. Physiol.,* **57**: 201–207.

Goldfarb, S., and T. Barka, 1960. Cytological localization of β-glucuronidase. *J. Histochem. Cytochem.,* **8**: 226–227.

Gomori, G., 1952. Microscopic Histochemistry. Chicago: University of Chicago Press.

Guardabassi, A., and E. Ferreri, 1953. Istofisiologia dell'apparato digerente di *Helix pomatia. Arch. Zool. Ital.,* **38**: 63–156.

Hess, R., and A. G. E. Pearse, 1958. The histochemistry of indoxylesterase of rat kidney with special reference to its cathepsin-like activity. *Brit. J. Exp. Path.,* **39**: 292–299.

Hirsch, G. C., 1915. Ernährungsbiologie fleischfressender Gastropoden. *Zool. Jahrb. Physiol.,* **35**: 357–504.

Holden, M., and M. V. Tracey, 1950. A study of enzymes that can break down tobacco-leaf components. 2. Digestive juice of *Helix* on defined substrates. *Biochem. J.,* **47**: 407–414.

Holt, S. J., 1956. The value of fundamental studies of staining reactions on enzyme histochemistry with reference to indoxyl methods for esterases. *J. Histochem. Cytochem.,* **4**: 541–554.

Jarrige, P., and R. Henry, 1952. Étude de l'activité glucuronidasique du suc digestif d'*Helix pomatia* L. et de sa phénolsulfatase. *Bull. Soc. Chim. Biol.,* **34**: 872–885.

Jeuniaux, C., 1954. Sur la chitinase et la flore bactérienne intestinale des mollusques gastéropodes. *Bull. Acad. Roy Belg.,* **CI**, Sci. Mem. 28. F7.

Jordan, H. J., and H. Begemann, 1921. Ueber die Bedeutung des Darmes von *Helix pomatia. Zool. Jahrb. Physiol.,* **38**: 565–582.

Krijgsman, B. J., 1925. Arbeitsrhythmus der Verdauungsdrüsen bei *Helix pomatia.* I. *Zeitschr. vergl. Physiol.,* **2**: 264–296.

Krijgsman, B. J., 1929. Arbeitsrhythmus der Verdauungsdrüsen bei *Helix pomatia.* II. *Zeitschr. vergl. Physiol.,* **8**: 187–280.

Lehrer, G. M., and L. Ornstein, 1959. A diazo coupling method for the electron microscopic localization of cholinesterase. *J. Biophys. Biochem. Cytol.,* **6**: 399–406.

Nakazima, M., 1956. On the structure and function of the midgut gland of mollusca, with a general consideration of the feeding habits and systematic relations. *Jap. J. Zool.,* **11**: 469–566.

Novikoff, A. B., 1959. Lysosomes and the physiology and pathology of cells. *Biol. Bull.,* **117**: 385.

Novikoff, A. B., and E. Essner, 1960. The liver cell: some new approaches to its study. *Amer. J. Med.,* **29**: 102–131.

Rose, G. G., 1957a. Microkinetospheres in VP satellites of pinocytic cells observed in tissue culture of Gey's strain HeLa with phase contrast cinematographic techniques. *J. Biophys. Biochem. Cytol.,* **3**: 697–704.

Rose, G. G., 1957b. Observations on the dynamics of pinocytic and varient pinocytic (VP) cells in Gey's human malignant epidermoid strain HeLa. *Texas Repts. Biol. Med.,* **15**: 313–331.

Rosen, B., 1941. Intrazelluläre Verdauung bei *Helix pomatia* L. *Zool. Jahrb. Abt. f. allg. Zool. u. Physiol.,* **60**: 241–252.

Rosenbaum, R. M., 1962. The intracellular localization of enzymes. *Handbk. Histochem.* VII, **4**: 1–108.

Rosenbaum, R. M., and C. I. Rolon, 1960. Intracellular digestion and hydrolytic enzymes in the phagocytes of planarians. *Biol. Bull.,* **118**: 315–323.

Rosenbaum, R. M., and C. I. Rolon, 1962. Species variability and the substrate specificity of intracellular acid phosphatases: a comparison of the lead-salt and azo-dye methods. *Histochem.,* **3**: 1–16.

Rosenbaum, R. M., and M. Wittner, 1962. Intracytoplasmic particles and enzymes associated with feeding and digestion in *Paramecium caudatum.* The possible role of the neutral red granules. *Arch. f. Protistenk.,* **106**: 223–240.

SEAMAN, G. R., 1961. Acid phosphatase activity associated with phagotrophy in the ciliate *Tetrahymena*. *J. Biophys. Biochem. Cytol.,* **9**: 243–245.

STRAUS, W., 1959. Regional cytochemical identification of phagosomes in various tissues of the rat and their differentiation from mitochondria by the peroxidase method. *J. Biophys. Biochem. Cytol.,* **5**: 192–204.

STRAUS, W., 1961. Cytochemical observations on the transport of intravenously injected horse-radish peroxidase and the development of phagosomes in the cells of the kidney of the rat. *Exp. Cell Res.,* **22**: 282–291.

THIELE, G., 1953. Vergleichende Untersuchungen über den Feinbau und die Funktion der Mitteldarmdrüse einheimischer Gastropoden. *Zeitschr. Zellforsch.,* **38**: 87–138.

VAN WEEL, P. B., 1961. The comparative physiology of digestion in molluscs. *Amer. Zool.,* **1**: 245–252.

WEISS, L. P., AND D. W. FAWCETT, 1953. Cytochemical observations on chicken monocytes, macrophages and giant cells in tissue culture. *J. Histochem. Cytochem.,* **1**: 47–65.

OSMOTIC REGULATION IN MARINE AND FRESH-WATER GAMMARIDS (AMPHIPODA)[1,2]

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Different species of the amphipod genus *Gammarus* live in habitats with a wide range of salt concentrations, from fresh water to sea water. To withstand such diverse conditions, the species must differ at least quantitatively in their osmotic physiology. In comparing four British species of the genus, Beadle and Cragg (1940a) found that the salinity range which each species tolerated correlated with the level at which it regulated the chloride concentration and the total osmotic concentration of its blood. More recently, Shaw and Sutcliffe (1961) and Lockwood (1961) have analyzed some mechanisms responsible for the osmoregulatory differences between two of Beadle and Cragg's species.

The present paper, too, attempts to analyze osmoregulatory mechanisms of gammarids, using an approach rather different from those of the previously mentioned investigators. The work involves four previously unstudied species: three in the genus *Gammarus* and one in the closely related genus *Marinogammarus*. The species are first compared with respect to the blood concentrations which they maintain in various dilutions of sea water. Then the role of the nephridium in salt and water excretion is studied in two species: one marine and one fresh-water. The results obtained imply some specific differences in the ionic uptake mechanisms.

EXPERIMENTAL MATERIAL

Adult males of the following four species were used:

Gammarus oceanicus Segerstråle (1947), characterized in the original description as "mainly marine," is widely distributed along both shores of the more northern parts of the Atlantic Ocean. It also lives in brackish situations and has been found in the Gulf of Finland in salinities as low as 2.5‰. Originally described as a subspecies of *G. zaddachi* Sexton, it was elevated to specific rank by Kinne (1954). The animals used in my experiments (mostly 18 to 23 mm. in length and 110 to 220 mg. in weight) were collected from an intertidal freshwater seep on Cape Cod Bay, about two miles north of the Cape Cod Canal.

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[2] Contribution number 1291, Woods Hole Oceanographic Institution.

[3] Part of the work reported here was undertaken while I was recipient of a National Science Foundation Graduate Fellowship, and part while I was research assistant at the Woods Hole Oceanographic Institution. I wish to express special indebtedness to my thesis director, Professor G. E. Hutchinson, whose suggestions led to the present researches, and to Mr. H. J. Turner, who made available the facilities of his laboratory at the Woods Hole Oceanographic Institution and who aided the work in innumerable ways.

Marinogammarus finmarchicus (Dahl) was characterized by Sexton and Spooner (1940, p. 659) as "a marine littoral species, occurring intertidally along the sea coast and for a short distance up estuaries." The genus was formerly (Schellenberg, 1937) a subgenus of *Gammarus*. The animals (about 15 to 20 mm. long) were collected from a rocky shore at Manomet, Massachusetts, on Cape Cod Bay.

Gammarus tigrinus Sexton (1939) is a brackish-water species very similar in appearance to *G. fasciatus*. For a time the two names were considered synonymous. That the forms are actually separate species is indicated by the following evidence: there are some slight but consistent morphological differences (Bousfield, 1958); the osmotic physiology of the two forms differs, as shown later in this paper; and in the culture experiments I performed, the two bred separately but did not cross-breed. The experimental animals came from a fresh-water seep high in the intertidal zone on Nonamesset Island, in Vineyard Sound, Massachusetts. They were 10 to 13 mm. long.

Gammarus fasciatus Say is the common fresh-water species in New England and was taken from various streams near Woods Hole, Massachusetts, and New Haven, Connecticut. The animals were 10 to 15 mm. in length and 25 to 60 mg. in weight.

METHODS

Determining osmotic concentration

The osmotic concentration of fluids was determined from the freezing point depression. Actually, the melting point of solutions was determined by observing a frozen sample as it was slowly warmed.

The apparatus used is a modification of the one designed by Kinne (1952) and is also similar to one devised by Ramsay (1949). It consisted of an aquarium of copper and glass which was mounted in the front wall of an insulated box and filled with a 25% solution of ethyl alcohol in water. The continuously stirred bath was warmed by an immersion heater, which was regulated by a variable transformer. The bath was cooled by a pack of crushed dry ice, which was pressed against the back of the aquarium by a spring-driven plunger. By careful balancing of the heat source and sink the temperature could be accurately controlled. The temperature was read with a Heidenhain thermometer graduated to 0.01° C.

To obtain a blood sample, the animal was gently dried off between layers of absorbent paper toweling, and its back was wiped with filter paper moistened with distilled water. With the animal held under paraffin oil the exoskeleton over the heart was punctured with a fine needle. From the drop of blood which appeared, a sample was taken with a fine Pyrex capillary tube, which had been cleaned in hot nitric acid. First, paraffin oil was drawn up into the capillary, then a small sample, and finally more paraffin oil. About a centimeter of capillary containing the sample was broken off, the ends sealed with sealing wax, and a label affixed. The samples were stored in a freezer. The size of the sample drops was not precisely controlled, but usually was 0.1 to 0.2 mm. in diameter and 0.3 to 1 mm. in length. This gave a range in volume from about 0.003 to 0.03 mm^3.

To determine the freezing point the sample was first frozen in crushed dry ice and then transferred to the controlled temperature bath, which had been cooled below the expected freezing point of the sample. As many as six samples were held simultaneously in the bath by a movable clamp. The samples were illuminated by light passed through a square of Polaroid film and reflected from a mirror behind them. They were watched through the front glass wall of the aquarium with a horizontally mounted microscope containing a Polaroid analyzer. Since ice crystals are birefringent, use of polarized light made the crystals easier to see as brilliant white objects against a dark background.

The temperature of the bath was raised fairly rapidly until most of the ice in the sample had melted. The rate of temperature rise was then slowed to 0.01° C. per minute, or less. At this slow rate it could be assumed that the ice and the melted solution were nearly in thermodynamic equilibrium, and that the last crystal disappeared at the freezing point, provided that the rate of temperature rise had been slowed five minutes or more before this event. The method was accurate within 0.01° C. on standard salt solutions. In duplicate blood samples from a series of animals the maximum difference in the freezing point was 0.02° C.

Since a major concern was with the osmotic movement of water, it was deemed most suitable to express the freezing point depressions and the salinities of the media as moles of ideal non-electrolytic solute per kilogram of water. The equivalent in moles of monovalent salt, such as NaCl, may be found by dividing by 2. Use of molal units has the additional convenience that the osmotic concentration of "normal" sea water (chlorinity of 19.4‰) is nearly unity: 1.03 molal. Hence the molality of a solution is nearly equal numerically to its fraction of sea-water strength.

Determining osmoregulatory behavior of species

Animals were individually isolated without food in about 200 ml. of medium in one-pint polyethylene boxes during the period of adaptation to a new salinity. It was experimentally determined that the blood concentration of *Gammarus oceanicus* reached a new steady-state about 12 hours after transfer from undiluted sea water (0.93 molal at Woods Hole) to 0.1 molal sea water. *G. fasciatus* reached a new steady-state within 1½ hours after transfer from fresh water to 0.6 molal sea water, the highest concentration in which it normally survived. Although adaptation times to other salinities were not determined, these experiments are taken to indicate that the one- or two-day period of adaptation was appropriate.

Animals were transferred to fresh water and to sea water of molality 0.03, 0.1, 0.2, 0.4, 0.61, 0.82, 0.93 or 1.03, and, in some instances, 1.5. (These concentrations correspond to salinities of 1.0, 3.5, 7.0, 14, 21, 28, 32 or 35, and 51.5‰.) All the species were exposed to the experimental media for 48 hours, or a little longer, with the exception of *G. oceanicus,* which was exposed for only 24 hours. Owing to the method of sampling, each animal could be used only once; hence the data shown on the curves are composite. The total number of surviving animals, distributed more or less equally among the various media, was 51 for *G. oceanicus,* 74 for *M. finmarchicus,* 77 for *G. tigrinus,* and 64 for *G. fasciatus.* The temperature range during the experiments was 16 to 19° C. for *G. oceanicus,* 14 to 16° C. for *M. finmarchicus* and *G. tigrinus*, and 13 to 18° C. for *G. fasciatus.* The curves were determined in July, 1955, for *G. oceanicus,* in September, 1955, for *G. fasciatus,* and in July, 1956, for *G. tigrinus* and *M. finmarchicus.*

No attempt was made to determine or control the molting stage of the experimental animals. Baumberger and Olmsted (1928) found that the concentration of the blood in some brackish-water crabs doubled during a molt. In my experiments the blood concentration in any one medium varied only slightly. Either a concentration change does not occur during molt in these animals or it is transitory and was not encountered.

Determining urinary rate and concentration

The structurally simple nephridium of *Gammarus* has an end-sac and a more or less coiled canal but no storage bladder (Burian and Muth, 1924; Schwabe, 1933). This arrangement should result in a continuous flow of urine, rather than intermittent micturition. The nephridium opens at the tip of a protuberance (the nephrocone) on the second segment of the second antenna; consequently the opening is readily accessible.

Sampling the urine was first attempted by immersing the animal in paraffin oil and, with a capillary tube, picking up drops of urine formed at the nephropore. This method was satisfactory for getting urine samples for freezing point determination, but not for finding rates of urine flow. The animals died after a short period under these conditions, presumably because the water clinging to the gills rapidly became anaerobic.

To overcome this difficulty the following arrangement was devised. A short, bent piece of glass tubing was cemented to the bottom of a Petri dish, with one end opening horizontally and the other end vertically. The dish was filled with water of the desired concentration to a depth that covered the horizontal end of the tubing. The animal was removed from the adapting medium, grasped firmly by the coxal plate of the first right thoracic leg with watchmaker's forceps, and backed gently into the open end of the tube. The forceps was then clamped in place. The coxal plate is extremely thin, but is broad and usually is quite hard and sturdy. Grasping it had no apparent effect upon the rate of urine flow. Various diameters of glass tubing were used so that the animals of different sizes could be fitted snugly. Thus, although the animal was held firmly at only one point, its movement was greatly restricted and it could not rotate about that point. After the animal had been introduced into the tube and had quieted, the water surrounding the tube was removed and replaced with paraffin oil. Surface tension held the water in the tube during the change, which left the animal with most of its body in water and only its head protruding into the oil. The water was aerated by a bubbler made of fine tubing inserted into the upper end of the tube.

Once the head and the nephrocones on the second antennae were surrounded by oil, it was possible to collect urine. For this purpose micropipettes were made from 0.3- or 0.5-mm. bore capillary tubing, tapered at the tip and graduated with strips of millimeter graph paper. The pipettes were filled with oil to avoid a strong capillary pull. They were then immersed in the oil bath with their tips capping the nephrocones. In some cases, especially with the smaller animals, the surface tension at the oil-urine interface of the minute nephridial opening apparently was sufficient to prevent the urine from flowing. To start the flow, it was usually necessary merely to touch the inside wall of the pipette to the nephropore and thereby break the interface. The rate of urine flow was determined from the progress of the urine drop up the pipette. Both nephridia were sampled simultaneously;

their rates rarely differed by as much as a factor of two. Urine flow was measured for about half an hour, a reading of the level in the pipettes being taken every five minutes in most cases, every two minutes in some others. The mean rates of urine flow for each side were added to give the total rate.

One difficulty was encountered with this method. Although most animals produced a measurable amount of urine when in any of the lower salinities, some did not. In most cases these animals had been injured in handling. Recently molted animals with soft exoskeletons were especially prone to damage. The results from damaged animals were discarded. There were a few cases of no urine flow for which there was no observed damage. Data from these animals are included in the graphs but not in the statistical calculations.

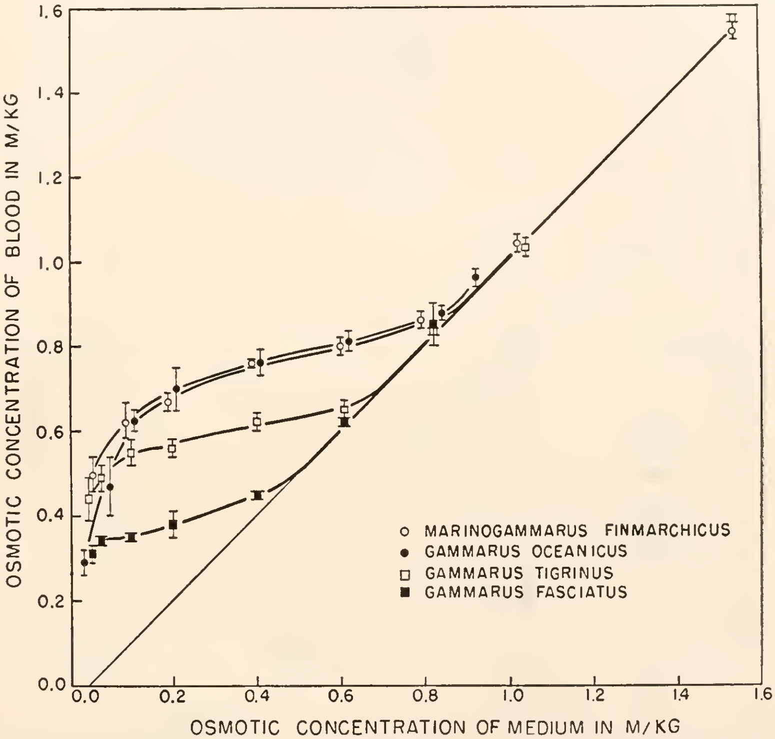

FIGURE 1. Relationship of concentration of blood (in moles of ideal solute per kg. of water) to concentration of medium in *Marinogammarus finmarchicus* and *Gammarus oceanicus* (marine), *G. tigrinus* (brackish-water), and *G. fasciatus* (fresh-water). Points represent means; vertical bars represent ±2 × the standard errors.

The choice of media was determined by the desire to find the relation of the rate of urine flow not only to the external concentration, but also to the osmotic gradient (obtained by subtracting the sea-water concentrations from the blood concentrations in Figure 1). The media chosen were ones in which a wide range of gradients would be expected: 0.03, 0.2, 0.61, and 0.93 molal sea water for the marine species *G. oceanicus,* and fresh water, 0.2, and 0.61 molal sea water for the fresh-water *G. fasciatus.* The experiments were carried out at approximately 15° C.

Results

Osmoregulatory behavior

The four species regulated osmotically in the manner shown in Figure 1. Each point is the mean of determinations on several animals (see methods section). The vertical lines at each point give ±2 × the standard error of the mean, or the 95% confidence interval for the mean.

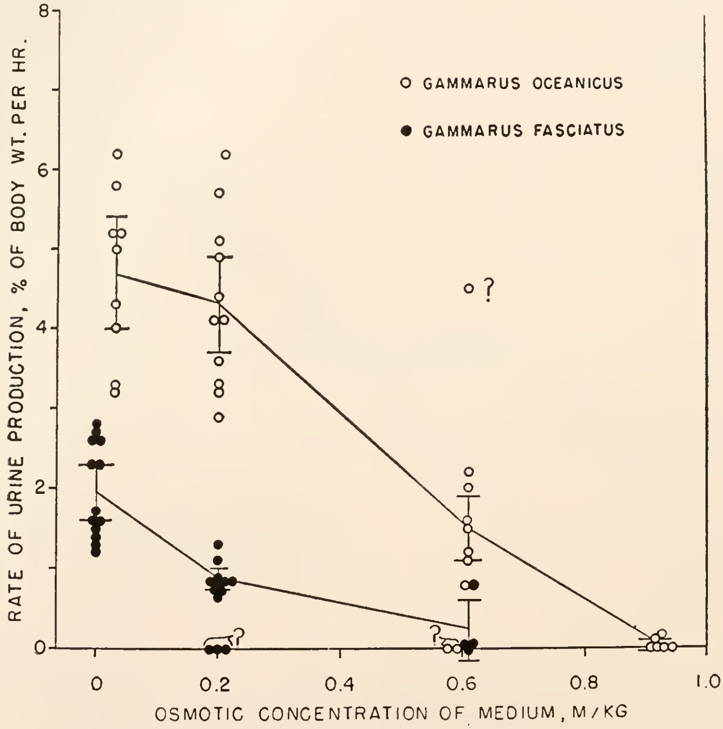

FIGURE 2. Relationship of rate of urine flow to external concentration in *Gammarus oceanicus* (marine) and *G. fasciatus* (fresh-water). Points represent individual animals. Points marked with a question mark are not included in the statistics. Curves connect the means. Vertical lines show ±2 × the standard errors.

The marine species (*M. finmarchicus* and *G. oceanicus*) exhibited nearly identical relationships of blood concentration to external concentration. In full strength sea water their blood was nearly isotonic to the medium. They began to regulate the blood concentration when sea water was diluted only slightly to below 0.85 molal. As the medium was further diluted, they maintained a progressively greater concentration gradient, and in 0.2 molal sea water the blood concentration was still 0.7 molal. In the most dilute sea-water media the gradient failed to increase further, or even decreased, but all animals in 0.03 molal sea water survived. In fresh water all *M. finmarchicus* died, but three out of ten *G. oceanicus* survived 24 hours, or long enough to be sampled, although their condition was probably moribund. In another experiment no *G. oceanicus* survived even this long in fresh water.

The brackish-water *G. tigrinus* displayed an osmotic behavior intermediate between that of *G. oceanicus* and *M. finmarchicus* on the one hand and that of *G. fasciatus* on the other. It regulated in media of less than 0.65 molal concentration and kept the blood concentration at 0.55 molal in 0.1 molal sea water and at 0.44 molal in fresh water. The most remarkable aspect of this experiment is that the species survived after direct transfer both to fresh water and to 1.5 molal sea water, although its mortality in either of these extreme concentrations was about 50%. In other experiments, some individuals survived a period of weeks in fresh water.

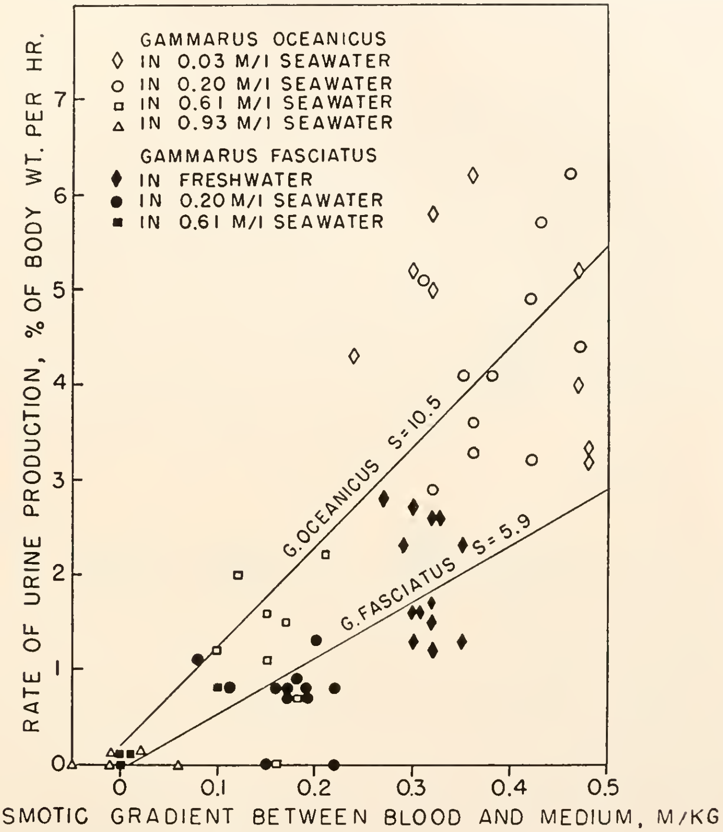

FIGURE 3. Relationship of rate of urine flow to osmotic gradient between blood and medium in *G. oceanicus* and *G. fasciatus*. Slope (S) determined by method of least squares.

G. fasciatus had a blood concentration of a little over 0.3 molal in fresh water, its normal habitat. When the animals were transferred into dilutions of sea water, the blood concentration increased only slightly and became isotonic in media with a concentration more than 0.5 molal. The species survived well in 0.6 molal sea water, but most individuals in 0.8 molal and all in 1.0 molal sea water died.

Urinary rate

Rate of urine flow is plotted against concentration of medium in Figure 2. Despite considerable variability in the data, it is apparent that in both species the rate of flow was much greater in the more dilute media. Urine production was detectable at higher salinities in *G. oceanicus* than in *G. fasciatus*. Moreover, the maximum rate (over 4% of the body weight per hour) in *G. oceanicus* was more than twice as great as the maximum rate (around 2%) in *G. fasciatus*.

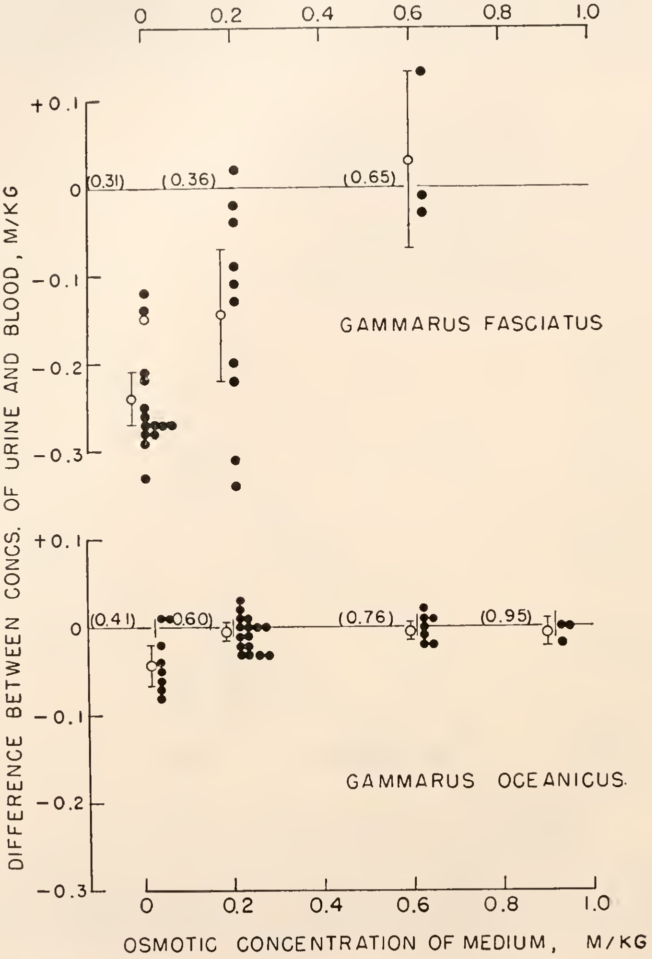

Figure 4. Relationship of urine concentration to blood concentration in various media, in *G. oceanicus* and *G. fasciatus*. Filled circles represent the difference: (urine concentration minus blood concentration) for individuals. Open circles show the mean difference; vertical lines ±2 × the standard errors. Mean blood concentrations are given in parentheses.

A few determinations on specimens of both species at 25° C. indicated that the rate was roughly twice that of animals in the same concentrations at 15° C.

The rate of urine flow in the two species is plotted against the osmotic gradient in Figure 3, and the regression lines are shown. The correlation coefficients for these data were 0.84 for *G. oceanicus* and 0.75 for *G. fasciatus*. In both cases the probability that a coefficient this size arose by chance is less than 0.001, as determined by a t-test. The slopes or regression coefficients show that the best estimate of the rate of urine production was 10.5% of the body weight per hour per molal gradient in *G. oceanicus* and 5.9% in *G. fasciatus*. If the regression coefficients are compared by a t-test presented by Fisher (1950), it is found that the probability they were drawn from the same statistical population is approximately 0.02.

Urine concentration

The difference between the urine concentration and the blood concentration for the two species of *Gammarus* in different salinities is shown in Figure 4. The urine of *G. oceanicus* was approximately isotonic to the blood in all media except the most dilute. In the latter, it was hypotonic by a small but statistically significant amount. The urine of *G. fasciatus* in fresh water was strongly hypotonic to the blood. In 0.2 molal sea water there was a great deal of variability, the urine ranging from equal in concentration to the blood to more dilute than the medium. The data for 0.6 molal sea water unfortunately are rather inadequate, partly because of difficulty in getting enough urine for a freezing point sample and partly because one of the samples seems to have been contaminated. However, the urine at this salinity appeared to be isotonic with the blood.

Discussion

Role of nephridium

Since the rate of water movement across a semipermeable membrane is proportional to the osmotic gradient, the demonstration that the urinary rate is proportional to the concentration difference between blood and medium supports the hypothesis that the water which enters *Gammarus* by osmosis is eliminated as urine. However, in order to prove the hypothesis completely, it would be necessary to measure the osmotic uptake of water by some independent method, and to demonstrate that urine flow is adequate to account for the removal of this water. Although this plausible mechanism of water balance in crustaceans has often been proposed before, the evidence regarding both the proportionality of urine flow and its adequacy has not been conclusive. The evidence on each of these points will be considered in turn in the following paragraphs.

In most studies of urinary rate in crustaceans and its relation to the external concentration, the method has been to plug the nephridial opening and to assume that subsequent weight increases represented normal urine formation. This procedure is suspect *a priori* in an armored animal such as a crustacean. One would expect blocking the nephridial opening to cause an increase in the hydrostatic pressure in the nephridium and, if urine formation occurs through filtration, to cause a change in the rate of this process. Indeed, plugging the kidneys has been reported as leading to the death of the experimental animals by Herrmann (1931) in *Astacus;* and by Nagel (1934) in *Carcinus*. While this method has sometimes yielded results in accord with those from other methods (see discussion of Parry's work, below), it has also given ambiguous results. For example, Nagel (1934) found that when crabs with plugged nephridia were transferred to brackish water they gained slightly more on the average than did similar crabs left in sea water. However, t-tests upon Nagel's data show the difference between transfer and control groups was of dubious statistical significance: $p > 0.2$ for the difference between rates for two groups given the same period of exposure; $p = 0.05$ for the difference between rates for two groups given different exposure times.

The most thorough study of the influence of salinity variation upon the rate of urine production in crustaceans was made by Parry (1955) in the prawn, *Palaemonetes varians.* This investigator estimated the rate of urine flow from four independent measurements: (1) the time for clearance of injected dye, (2) the frequency of *micturition* from a bladder of known size, (3) the weight change after blockage of the nephropores, and (4) the volume of urine collected by cannulation of one of the nephropores. All four methods gave similar results. Parry did not relate the rates to the gradient between blood and medium, since she did not determine blood concentrations. However, if we use concentrations determined for the same species by Panikkar (1941; also cited by Parry), we can consider the data from this viewpoint (Table I). We see that during hypertonic regulation in external concentrations of less than about 0.6 mole/kg., the rate of urine production was approximately proportional to the osmotic gradient. It is conceivable that the small increase in urinary rate during hypotonic regulation has the same cause as the increased urinary rate in injured marine teleosts (Smith, 1932), namely, the necessity of excreting the extra divalent ions in swallowed sea water.

TABLE I

Relation between osmotic gradient and rate of urine flow in Palaemonetes varians

Osmotic conc. of medium (moles/kg.)	Gradient between blood and medium (calculated from Panikkar, 1941)	Urine flow as % of body wt./hr. (determined by rate of micturition, Parry, 1955)	Ratio: $\left(\frac{\text{Urine flow}}{\text{gradient}}\right)$
.05	.49	1.63	3.3
.15	.39	1.06	2.7
.25	.29	.94	3.2
.50	.07	.15	2.1
.67	−.10	.45	−4.5
.85	−.25	.40	−1.6
1.00	−.34	.42	−1.2
1.20	−.51	.11	− .22

The rate of water entry into crustaceans has not been determined with sufficient precision to permit an exact comparison with the rate of urine flow. In the previously mentioned study, Parry found that the half-time for penetration of heavy water into *P. varians* in nearly isotonic conditions was one-half to three-fourths hour. Such determinations may be used (*cf.* Lockwood, 1961) to predict the rate at which water should be absorbed under a specified osmotic gradient. Treating Parry's data in this manner, one may calculate that an amount equaling 1.9% to 2.5% of the total body water (or 1.5% to 2.0% of body weight, if a prawn is 80% water) should enter per hour and per gradient of one mole/kg. The values for urinary rates in column 4, Table I, are roughly comparable (2.1% to 3.3% of body weight per hour and per gradient of one mole/kg.).

Lockwood (1961) has determined the rate of penetration of tritiated water into two British species of *Gammarus: G. pulex* and *G. duebeni.* The difference between the two species was not statistically significant. The half-time for penetration in *G. duebeni* was 13.9 minutes, which leads to an estimated rate of water entry of about 4.7% of body weight per hour per gradient of 1 mole/kg. While this is less than the rates of urine flow shown by the slopes in Figure 3, it is of the same order of magnitude.

In summary, the similarity of observed urinary rates to those predicted from permeability studies is at least in accord with the view that osmotically absorbed water is eliminated as urine. The data are not sufficiently exact, however, to be decisive.

Comparison of species

A. Comparison of *G. fasciatus* and *G. oceanicus*

Three differences have been shown which give the fresh-water *G. fasciatus* a lower rate of urinary salt loss than the marine *G. oceanicus.* They are (1) a smaller concentration gradient between blood and medium, (2) a lesser permeability to water,[4] and (3) a urine which is hypotonic to the blood.

The first two differences result in a lower rate of urine flow; the third results in a smaller salt loss for a given volume of urine. The quantitative result of these differences is shown in Figure 5, in which the urinary rate is multiplied by the urine concentration to give the rate of salt loss in micromoles of solute particles per hour in a 100-mg. animal.

B. Comparison with other species

None of the species in this study was the same as any in the study by Beadle and Cragg (1940a). Two of their species are not known to occur at all in North America, and the other two do not occur in southern New England, where the present study was made. However, Beadle and Cragg's findings were similar to those presented here in that their more marine species, *G. locusta* and *G.* (*Marinogammarus*) *obtusatus,* regulated the blood concentration at a high level; the brackish-water *G. duebeni* regulated at an intermediate level; and the fresh-water *G. pulex,* at a low level. The major differences from the present study were that their marine species, especially *G. obtusatus,* did not survive in nearly as dilute sea water as did *G. oceanicus* and *M. finmarchicus;* and that none of their species could survive in both fresh water and full strength sea water. (Later, however [1940b], Beadle and Cragg reported on a fresh-water race of *G. duebeni.*)

The recent papers of Lockwood (1961) and of Shaw and Sutcliffe (1961) were concerned largely with water entry, which has already been discussed, and with sodium uptake, which will be discussed in the next section. Several aspects of the salt loss remain to be compared. Shaw and Sutcliffe found that the rate of salt loss by all routes from a 40-mg. *G. duebeni* varied from 0.17 to 0.76 micromole of NaCl per hour, being less when the animal had been adapted to a more dilute medium. For *G. pulex,* the rate was 0.09 to 0.18 micromole per hour.

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[4] From the difference in slopes in Figure 3. The rates of urine flow in this figure are based on the weights of the animal, whereas permeabilities should be based on the surface areas, which are not known. It can easily be shown, however, that since *G. oceanicus* averaged about five times the weight of *G. fasciatus,* conversion of urinary rate to permeability would *increase* the difference found between the species.

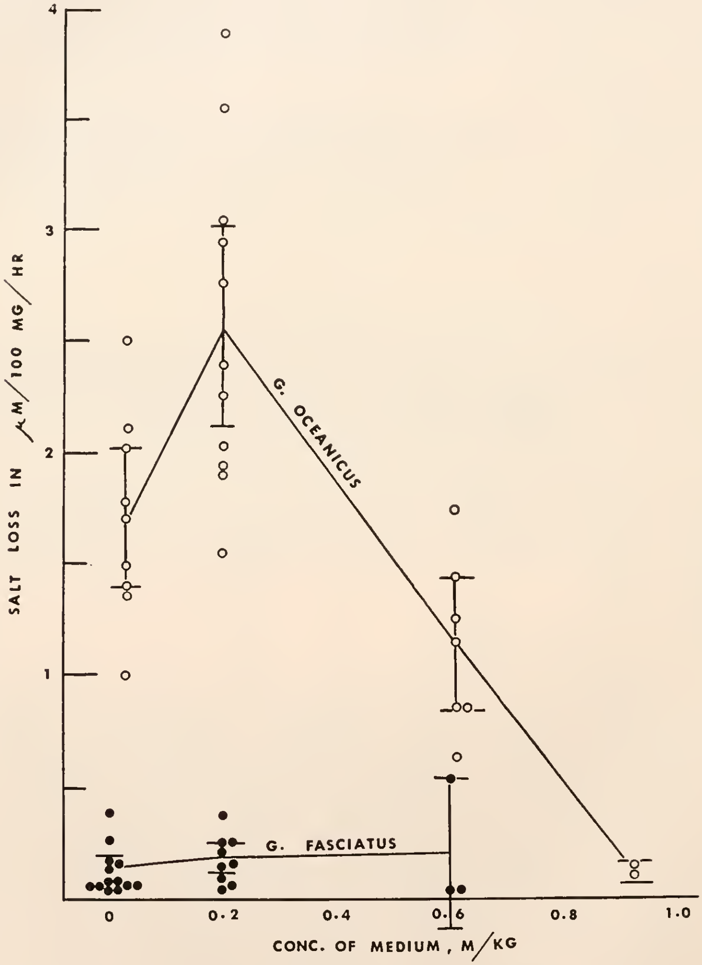

FIGURE 5. Comparison of rate of urinary salt loss in *G. oceanicus* and *G. fasciatus.* Points represent individuals. Curves connect means. Vertical lines show ±2 × the standard errors.

Lockwood determined that in very dilute sea water both the brackish-water *G. duebeni* and the fresh-water *G. pulex* formed urine hypotonic to the blood. In the higher concentrations of sea water the urine of *G. duebeni* became isotonic to the blood, but the urine of *G. pulex* remained hypotonic to the blood and also to the medium. Estimating the urine flow from the penetration of tritiated water, Lockwood calculated that only 0.10 micromole per hour of the NaCl loss from *G. duebeni* could be accounted for by the urine. Apparently there is a substantial loss by some other route, probably across the body surface.

From Figure 5 one can calculate that a 40-mg. *G. oceanicus* in the most dilute sea water would retain salt less efficiently than *G. duebeni* and would lose about 0.34 micromole of NaCl per hour through the urine alone. A *G. fasciatus* of the same size in fresh water would lose only about 0.02 micromole per hour.

Mechanism of salt uptake

The differences demonstrated between *G. fasciatus* and *G. oceanicus* bring to the fore a curious problem. The steady-state osmotic gradient maintained by these animals represents the conditions at which salt loss by all routes just equals salt uptake. Since the urine concentration and flow rate are more favorable for osmotic regulation in *G. fasciatus* than in *G. oceanicus*, how can the latter maintain a much greater osmotic gradient when both species are in the same medium? One logical possibility is that *G. fasciatus* is more permeable to salt than *G. oceanicus* and loses more salt by diffusion. This would be contrary to the results of other investigations of the comparative permeabilities of marine, brackish-water, and fresh-water crustaceans (Gross, 1957; Bethe, 1930; Nagel, 1934). The only alternative explanation of the apparent paradox is that when both species are in the same medium, the rate of salt uptake is greater in *G. oceanicus*.

From the steady-state condition it is apparent that when a gammarid is in a high external concentration, the mechanism for salt uptake must be operating either not at all or at only a very low rate, since the gradient is zero. The gradient becomes appreciable—that is, uptake begins—only when the concentration is brought below a certain critical level. Activation of the mechanism must be gradual, since the osmotic gradient increases gradually as concentration is further lowered. The critical concentration at which uptake and regulation begin is characteristic of the species, being highest in the marine species (*G. oceanicus* and *M. finmarchicus*), next highest in the brackish-water species (*G. tigrinus*), and lowest in the fresh-water species (*G. fasciatus*). Consequently, in most salinities the uptake mechanism is more completely activated in *G. oceanicus* than in *G. fasciatus* and the former species maintains a greater gradient. The degree of activation of the uptake mechanism might depend upon either the concentration of the medium or that of the blood. The latter alternative is favored by Shaw's (1959) demonstration that in the crayfish, sodium uptake from fresh water increases with decreasing blood concentration.

In most dilute media the gradient maintained by a species falls below the maximum, indicating a drop in the salt uptake rate. In this circumstance *G. oceanicus* no longer has a gradient greater than *G. fasciatus;* indeed, in fresh water it cannot maintain an internal concentration sufficiently high for survival.

Shaw and Sutcliffe (1961) directly measured uptake by *Gammarus* from very dilute media and found that with increasing external concentration the uptake rate rose asymptotically to a maximum. Since this behavior can be described by the Michaelis equation for effect of substrate concentration on rate of an enzyme-intermediated reaction, the implication is that in very dilute media the uptake mechanism becomes unsaturated. Using this interpretation, Shaw and Sutcliffe further concluded that the uptake mechanism of *G. pulex* had a greater affinity for ions than that of *G. duebeni,* and therefore the former could maintain a greater gradient in the most dilute media. This analysis would equally well explain the difference in osmotic behavior between *G. fasciatus* and *G. oceanicus.*

SUMMARY

1. The relationship of the osmotic concentration of the blood to that of the external medium is described for four species of gammarid. In media of various concentrations each species regulates its blood concentration in a manner that reflects its natural habitat. The marine species, *Marinogammarus finmarchicus* and *Gammarus oceanicus,* regulate their blood concentration at the highest level; the brackish-water species, *G. tigrinus,* regulates at a lower level; and the fresh-water species, *G. fasciatus,* regulates at the lowest level. Moreover, *M. finmarchicus* and *G. oceanicus* die in fresh water; *G. fasciatus* dies in full-strength sea water; but *G. tigrinus* survives both in fresh water and in sea water up to at least 1.5 molal.

2. The rate of urine production in *G. fasciatus* and *G. oceanicus* is proportional to the osmotic gradient between blood and medium, indicating that urine formation represents elimination of osmotically absorbed water. The coefficient of proportionality is smaller in *G. fasciatus* (5.9% of body weight per hour per molal gradient) than in *G. oceanicus* (10.5%), indicating that the latter species is more permeable to water.

3. The urine of *G. oceanicus* is nearly isotonic to the blood in all media. The urine of *G. fasciatus* is much more dilute than the blood.

4. The differences in flow and in concentration of urine combine to give *G. oceanicus* a much greater rate of urinary salt loss than *G. fasciatus.*

5. The osmotic gradient maintained by each species varies in a way that indicates the animals have an ionic uptake mechanism which is gradually activated as the salinity is lowered. In all except the most dilute media, it appears that the mechanism is more completely activated and takes up salt more rapidly in *G. oceanicus* than in *G. fasciatus.*

LITERATURE CITED

BAUMBERGER, J. P., AND J. M. D. OLMSTED, 1928. Changes in the osmotic pressure and water content of crabs during the molt cycle. *Physiol. Zoöl.,* **1**: 531–545.

BEADLE, L. C., AND J. B. CRAGG, 1940a. Studies on adaptation to salinity in *Gammarus* sp. I. Regulation of blood and tissues and the problem of adaptation to fresh water. *J. Exp. Biol.,* **17**: 153–163.

BEADLE, L. C., AND J. B. CRAGG, 1940b. Osmotic regulation in freshwater animals. *Nature,* **146**: 588.

BETHE, A., 1930. The permeability of the surface of marine animals. *J. Gen. Physiol.,* **13**: 437–444.

BOUSFIELD, E. L., 1958. Fresh-water amphipod crustaceans of glaciated North America. *Canad. Field-Nat.,* **72**: 55–113.

BURIAN, R., AND A. MUTH, 1924. Die Exkretion (Crustaceen). *In:* Winterstein's "Handbuch der vergl. Physiol." Bd. 2, 2. Hälfte, pp. 633–695.

FISHER, R. A., 1950. Statistical Methods for Research Workers. 11th ed. Hafner Publ. Co., New York.

GROSS, WARREN J., 1957. An analysis of response to osmotic stress in selected decapod crustaceans. *Biol. Bull.,* **112**: 43–62.

HERRMANN, F., 1931. Über den Wasserhaushalt des Flusskrebses (*Potamobius astacus* Leach). *Zeitschr. vergl. Physiol.,* **14**: 479–524.

Huf, E., 1936. Der Einfluss des mechanischen Innendrucks auf die Flussigkeitsausscheidung bei gepanzerten Süsswasser- und Meereskrebsen. *Pflüg. Arch. ges. Physiol.*, **237**: 240–250.

Kinne, O., 1952. Ein neues Gerät zur Bestimmung der Gefrierpunktserniedrigung kleiner Flüssigkeitsmengen. *Veröff. Inst. Meeresforsch., Bremerhaven.*, **1**: 47–51.

Kinne, O., 1954. Die *Gammarus*-Arten der Kieler Bucht. *Zool. Jahrb., Abt. Syst., Ökol., u. Geogr. Tiere,* **82**: 405–424.

Lockwood, A. P. M., 1961. The urine of *Gammarus duebeni* and *G. pulex*. *J. Exp. Biol.*, **38**: 647–658.

Nagel, H., 1934. Die Aufgaben der Exkretionsorgane und der Kiemen bei der Osmoregulation von *Carcinus maenas*. *Zeitschr. vergl. Physiol.*, **21**: 468–491.

Panikkar, N. K., 1941. Osmoregulation in some palaemonid prawns. *J. Mar. Biol. Assoc.*, **25**: 317–359.

Parry, G., 1955. Urine production by the antennal glands of *Palaemonetes varians* (Leach). *J. Exp. Biol.*, **32**: 408–422.

Ramsay, J. A., 1949. A new method of freezing point determination for small quantities. *J. Exp. Biol.*, **26**: 57–64.

Schellenberg, A., 1937. Schlüssel und Diagnosen der dem Süsswasser-Gammarus nahestehenden Einheiten ausschliesslich der Arten des Baikalsees und Australiens. *Zool. Anz.*, **117**: 267–280.

Schwabe, E., 1933. Über die Osmoregulation verschiedener Krebse (Malacostracen). *Zeitschr. vergl. Physiol.*, **19**: 183–236.

Segerstråle, S. G., 1947. New observations on the distribution and morphology of *Gammarus zaddachi* Sexton, with notes on related species. *J. Mar. Biol. Assoc.*, **27**: 219–244.

Sexton, E. W., 1939. On a new species of Gammarus (*G. tigrinus*) from Droitwich district. *J. Mar. Biol. Assoc.*, **23**: 543–552.

Sexton, E. W., and G. M. Spooner, 1940. An account of *Marinogammarus* (Schellenberg) gen. nov., with a description of a new species, *M. pirloti*. *J. Mar. Biol. Assoc.*, **24**: 633–682.

Shaw, J., 1959. The absorption of sodium ions by the crayfish, *Astacus pallipes* Lereboullet. I. The effect of external and internal sodium concentrations. *J. Exp. Biol.*, **36**: 126–144.

Shaw, J., and D. W. Sutcliffe, 1961. Studies on sodium balance in *Gammarus duebeni* Lilljeborg and *G. pulex pulex* (L.). *J. Exp. Biol.*, **38**: 1–15.

Smith, Homer W., 1932. Water regulation and its evolution in fishes. *Quart. Rev. Biol.*, **7**: 1–26.

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THE SIGNIFICANCE OF THE CAUDAL EPIDERMIS IN ASCIDIAN METAMORPHOSIS[1]

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Free swimming ascidian larvae normally attach to solid substrata before the onset of metamorphosis. Morphogenetic changes include tail resorption, reorientation of the digestive system, heart and adult neural tissues (or their primordia) and the formation of epidermal ampullae. Degeneration of the larval nervous and sensory systems and the loss of the outer layer of tunic follow rapidly.

The most astonishing event in metamorphosis is the withdrawal of the tail. In the simple ascidians, *Boltenia villosa, Pyura haustor, Styela gibbsii,* and *Styela partita,* the major part of tail resorption is completed within 10 minutes. In the compound ascidian *Amaroucium constellatum* the tail is completely withdrawn into the posterior region of the trunk in about 6 minutes. Histological studies of *Boltenia, Pyura* and *Styela* (Cloney, 1961a) have demonstrated that a disruption of the intercellular cementing substances or binding forces between the notochordal and muscle cells occurs during tail resorption. These disruptive changes begin proximally and progress distally as the tail shortens. The anterior end of the notochordal sheath first explosively ruptures. This allows the notochordal cells and the extracellular matrix of the notochord to flow into the posterior region of the trunk. Simultaneously the muscle cells buckle and their myofibrils become disarranged and are no longer oriented parallel to the axis of shortening. The nerve cord and endodermal strand are passive and become compressed as the tail shortens. The epidermis thickens but shows no signs of dissociating into separate cells. This pattern of tail resorption will be referred to as Type 1.

In *Ciona intestinalis* (Weiss, 1928), *Phallusia mammillata, Clavelina lepidiformis* (Berrill, 1947), *Amaroucium constellatum* (Scott, 1952), *Ascidia nigra* (Grave, 1935) and *Ascidia callosa* (Cloney, 1961a), the notochord-muscle-nerve cord complex (NMN-complex) remains intact as a unit as the caudal tissues are withdrawn. The NMN-complex separates from the epidermis and moves into the

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posterior region of the trunk. In some species the complex is formed into a helix as it moves. The epidermis finally forms a cap over the resorbed tissues. This will be called Type 2 tail resorption.

When tails of *Boltenia* larvae were excised before the onset of metamorphosis, the distal fragments remained alive and were observed to twitch or even swim about for one or two days after the operation, but they showed no signs of the histological changes associated with tail resorption. Connection with the trunk is normally essential for these changes.

If, however, the tails were excised through the proximal region of shortening after the onset of metamorphosis they could undergo shortening in isolation. Evidently the mechanism for tail resorption resides within the tail tissues after the process has begun. Isolated tail can also be induced to shorten with proteolytic enzymes.

Instructive cases of partial tail resorption in chloretone-treated *Ciona* larvae have been observed by Weiss 1928). In these specimens the NMN-complex remained rigid but the epidermis sometimes became torn or ruptured in places and pulled into a mass near the tip of the tail. Weiss emphasized that the epidermis can therefore undergo typical regressive changes by itself. Direct observations of tail resorption, however, led him to conclude that both epidermal and tonic muscular contraction are probably essential for complete tail involution in *Ciona*.

Berrill (1947) contended that tail resorption is caused principally by the shrinkage of the epidermis due to its so-called "nutritional exhaustion" in *Ciona, Ascidiella, Phallusia, Styela, Styelopsis, Distomus, Stolonica, Clavelina* and *Distaplia*. This opinion was supported by Scott (1952). Cloney (1961a) argued that the epidermis is an active tissue, as evidenced by (1) the resorption of the epidermal adhesive papillae within two to three minutes after the onset of metamorphosis in *Boltenia, Pyura, Styela* and *Ascidia;* (2) the formation of an invagination of the epidermis behind the resorbed tail tissue elements at the end of tail resorption, and (3) the rapid formation of the epidermal ampullae which spread out over the substratum shortly after attachment of the larva. There is no evidence of "nutritional exhaustion" in these species; indeed, the epidermal cells display considerable activity and they also contain yolk granules. (For a fuller discussion of this point see Cloney, 1961a.)

It was suggested that the release of a proteolytic enzyme in the posterior region of the trunk in *Boltenia* could account for the rupture of the notochord, the dissociation of the muscle and notochordal cells, and that the epidermis contracts actively as a unit, forcing the dissociated tissues into the trunk.

But, since in *Boltenia, Pyura,* and *Styela,* it was not possible to isolate or to observe the independent contraction of the tail epidermis, only indirect evidence for its active role in the resorption of the tail could be obtained. Consideration of Type 1 and Type 2 tail resorption led to the following questions: (1) Does contraction of the muscle cells play any part in the process? All analyses of tail resorption have shown that the epidermis is important, but (2) what is the mechanism of epidermal contraction? (3) What factors initiate and synchronize the observed histological changes with general metamorphosis? (4) Can the two distinct types of tail resorption be explained with a single hypothesis? This report on the compound ascidian, *Amaroucium constellatum,* provides direct evidence for:

(1) the active contraction of the epidermis in unaesthetized larvae, (2) the insignificance of the muscle cells in the overall mechanism of tail resorption, (3) a possible explanation for other histological changes in the caudal tissues of ascidians during tail resorption.

METHODS

Observations were made on larvae of the compound ascidian *Amaroucium constellatum* collected by the Supply Department in the vicinity of the Marine Biological Laboratory, Woods Hole, Massachusetts. Colonies were kept in running sea water in a light-tight box for 8 to 18 hours prior to the period when they were needed. Colonies were then exposed to light in a small dish of sea water. This method, used by Scott (1952), induces the release of swimming larvae in about 20 minutes. Observations of living animals were made with and without supported coverslips under a compound microscope equipped with NA 0.25 and 0.65 objectives. In some cases metamorphosis was stimulated with a 1:500,000 dilution of Janus green B. Cinematographic records were made of both normal tail resorption and the effects of experimental interference with tail resorption. Tissues were fixed in 2.7% OsO_4 buffered in 0.1 *M* *S*-collidine and embedded in Epon, according to the method of Luft (1961). Sections were cut at 1 μ and stained with Richardson's stain (Richardson *et al.*, 1960). Photomicrographs of sections were made with a Zeiss NA 1.25 planachromat objective.

RESULTS

A. *Structure of the larval tail*

Details of the larval anatomy have been described by Grave (1921) and Scott (1946). The following descriptions of the tail tissues are limited to details regarded as essential to a discussion of tail resorption.

Tunic. The entire trunk and tail of the larva is covered by two tough membranous layers of tunic. The outer layer forms the dorsal and ventral fins and is lost as a molt during metamorphosis. The inner layer is retained as part of the post-larval tunic. Free amoeboid cells are frequently found between the layers (Fig. 1). The fine structure of this complex structure will be described in a subsequent paper.

Epidermis. The epidermis is a simple squamous epithelium supported basally by a thick amorphous basement membrane which lies in close contact with the surfaces of the underlying muscle cells. In transverse sections of the tail the epidermis consequently tends to conform to the scalloped contours of the muscle bands (Fig. 1).

Muscle. Four rows of muscle cells are arranged in bands on the right and left sides of the tail. (Since the tail is twisted 90° to the left during development, the top of the tail is considered to represent the anatomical right side and the bottom the left side. The dorsal and ventral fins lie in the frontal plane.) Within each row, the muscle cells abut against each other at their ends without the intervention of a connective tissue septum. Each muscle cell is roughly cylindrical in shape. Contractile myofibrils lie immediately beneath the sarcolemma in a single layer. They spiral along the course of the cell at an angle of about 18° to the right (Grave, 1921). They are disposed entirely around the periphery of each cell except for

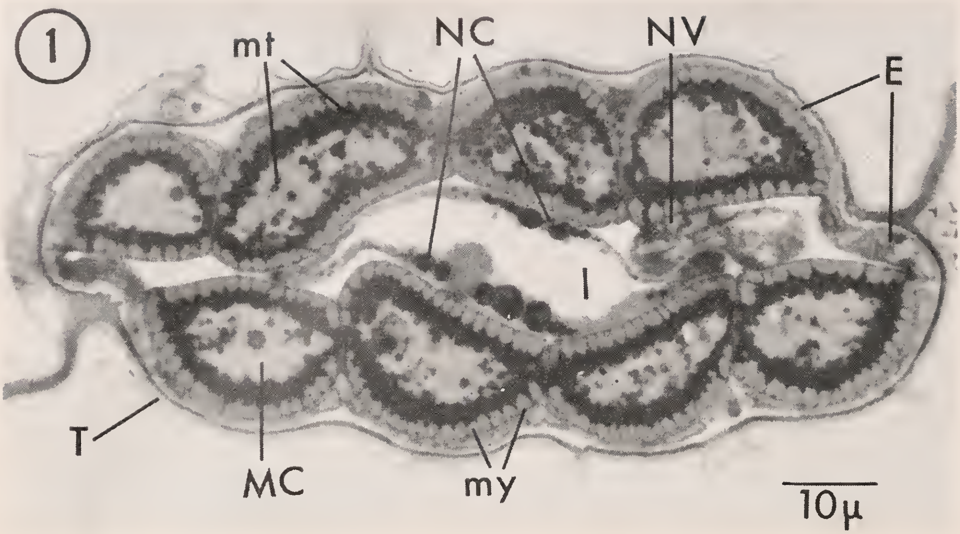


FIGURE 1. *Amaroucium constellatum;* transverse section of the larval tail. The tail is covered by two layers of tunic (T). The outer layer forms the dorsal and ventral fins. The basement membrane of the thin epidermis (E) lies in close contact with 8 rows of muscle cells (MC). Myrofibrils (my) lie in the periphery of each cell. They are underlain by a thick layer of mitochondria (mt). The muscle cells attach to a fibrous sheath of the notochord. Notochordal cells (NC) form a squamous epithelium beneath the sheath. The axis or lumen (l) of the notochord is filled with a clear matrix. The nerve cord (NV) is visible on the right. One-micron Epon section; Richardson's stain.

small gaps where adjacent muscle cells lie in close contact. Contrary to the opinion of Grave (1921), Scott (1946) and Berrill (1947), the myofibrils terminate near the sarcolemma at the ends of each cell (Jackson, 1958; Cloney, unpublished results). A dense layer of mitochondria is located beneath the myofibrils. The nucleus is located near the center of each cell and is surrounded by relatively clear cytoplasm. Lead acetate staining of ultrathin sections suggests the presence of glycogen in these areas. Large, irregular, dense bodies are often found in the cytoplasm of the muscle cells. In electron micrographs these bodies can be seen to contain myelinic figures. The band of muscle cells on the right side of the tail is often shifted slightly with respect to the left band of muscle, giving the tail an asymmetric appearance in section (Fig. 1).

Notochord. The notochord forms the axis of the tail. In transverse sections the notochord appears elongate in the dorsal-ventral axis and somewhat irregular in shape. In electron micrographs, it is seen to be surrounded by an acellular filamentous sheath. The cells of the notochord are arranged in an epithelium and are attached to the inside of the notochordal sheath. The cells contain both proteid and lipid yolk granules. The axis or lumen of the notochord is filled with a clear matrix. The matrix stains poorly and has low electron density. Processes frequently extend from the notochordal cells into the lumen (Figs. 1 and 2).

Nerve cord. The dorsal nerve cord has a well formed lumen in many places. The cord extends posteriorly from the visceral ganglion into the dorsal region of the tail. Nerve processes have not yet been observed passing from the cord to the muscle cells (Fig. 1).

B. *Tail resorption*

The basic morphological changes accompanying metamorphosis in *Amaroucium* have been described by Scott (1952). Only the details of tail resorption will be considered here.

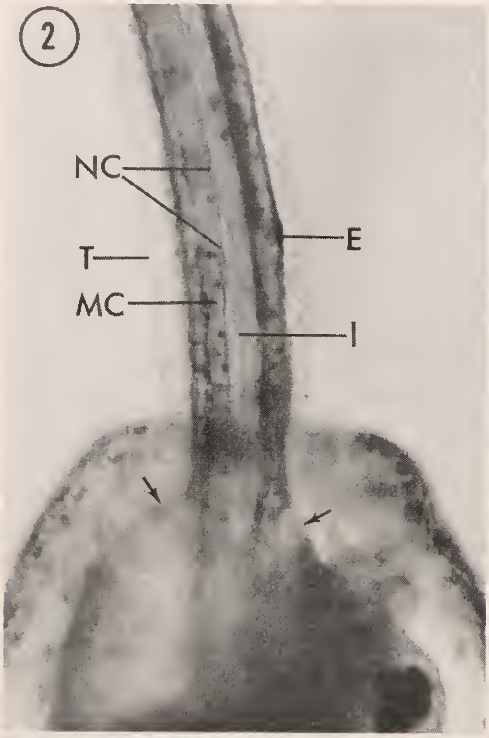
In 14 recorded cases, the first detectable morphological changes associated with tail resorption occurred on an average of two minutes, two seconds after the discharge of the larval adhesive papillae (see Table I). The discharge of a sticky substance by the papillae is typically associated with attachment in *Amaroucium,* but metamorphosis may ensue with or without attachment. Metamorphosis may be spontaneous or it may be induced by a heterogeneous variety of substances (Lynch, 1961). Changes in the tail tissues begin with the lifting away of the epidermis from the underlying notochord-muscle-nerve cord (NMN) complex with the formation of a subepidermal fluid-filled space (Figs. 3, 6). Simultaneously the notochord loses its turgidity. This results in a partial collapse of the tail (Fig. 3). The notochordal cells begin to round up and large vesicles sometimes become visible within the matrix of the notochord (Fig. 6). Under favorable optical conditions these vesicles can sometimes be seen to flow toward the trunk. The entire NMN-com-

TABLE I

Timing of tail resorption.

The first sign of the onset of metamorphosis in A. constellatum is the discharge of the adhesive papillae. In column I, tabulations indicate the elapsed time in minutes and seconds from the discharge of the adhesive papillae to the first visible changes in the tail. Column II indicates the time elapsed from the earliest changes in the tail to the completion of tail resorption. Column III indicates the total elapsed time from the discharge of the adhesive papillae to the completion of tail resorption. Measurements were made at 24.5°C. Tabulations of 14 separate cases were arranged in order of increasing total time.

Specimen	Discharge of papillae to beginning of tail resorption I	Beginning to completion of tail resorption II	Total time III
1	2′20″	4′00″	6′20″
2	1′10″	5′40″	6′50″
3	2′15″	5′00″	7′15″
4	1′56″	5′37″	7′33″
5	1′50″	5′45″	7′35″
6	2′25″	5′10″	7′35″
7	1′25″	6′20″	7′45″
8	2′42″	5′05″	7′47″
9	2′30″	6′00″	8′30″
10	2′45″	5′50″	8′35″
11	1′45″	6′50″	8′35″
12	2′05″	6′32″	8′37″
13	2′00″	6′45″	8′45″
14	1′21″	7′53″	9′14″
Average	2′02″	5′54″	7′55″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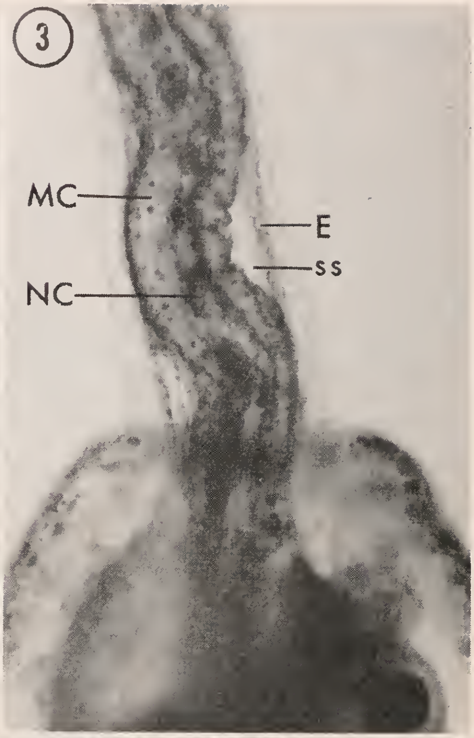
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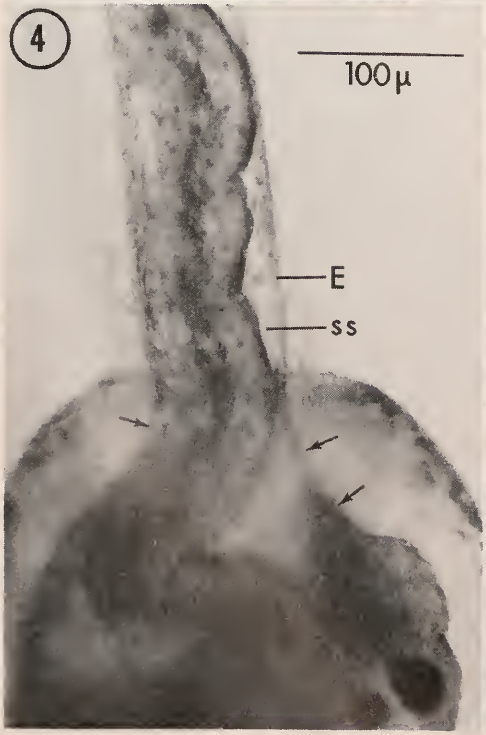
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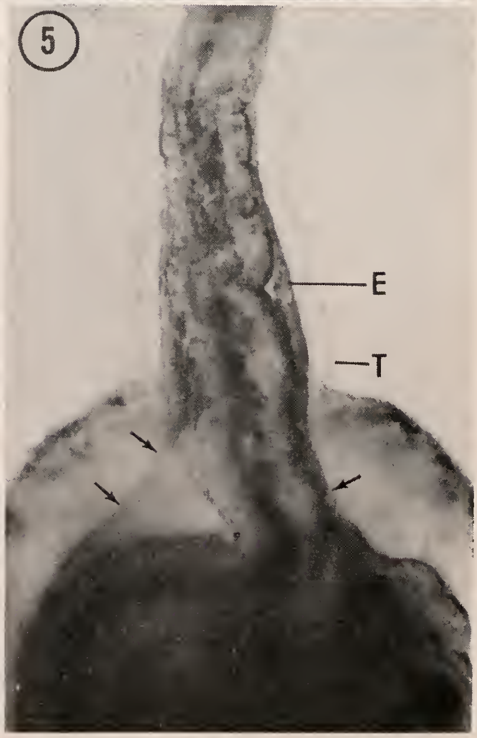
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plex beneath the epidermis begins to buckle and fold as it moves into the posterior region of the trunk (Figs. 3, 4, 5). The epidermis continuously thickens and appears to be under tension. The epidermis finally forms into a thick cap at the posterior end of the trunk, enclosing the entire NMN-complex within the body cavity.

As the tail tissues are withdrawn, the double-layered tunic which covers the tail becomes greatly folded. Near the completion of tail resorption the outer layer of tunic springs away from the underlying tissues and is pushed out, forming an empty sac. This outer layer drops away within a few hours as a cuticular molt. This outer layer need not be regarded as important to tail resorption because it can be pulled off with a pair of forceps before metamorphosis begins without affecting the process in any way.

In observing tail resorption, one might infer that the epidermis is actively contracting because it appears to be under tension during this period, but it is of course impossible to be certain of this from microscopic observations alone.

To test the theory of active epidermal contraction, the tails of larvae *which had just begun to metamorphose* (after the epidermis has separated from the underlying NMN-complex) were either excised about halfway along their length or they were regionally damaged by touching them with a needle. When this was done the epidermis immediately split into two units: an isolated distal piece and a proximal piece continuous with the trunk epidermis. *Immediately following this operation both the proximal and the distal pieces of epidermis began to shorten over the surface of the underlying NMN-complex* (Figs. 7–10). *Sometimes the epidermis contracted, pulling the underlying tissues with it for a short distance, and then the NMN-complex, evidently under tension, broke loose and straightened out again while the epidermis continued to contract.* The proximal epidermis shortened into a thick annular ring at the base of the tail while the distal fragment pulled distally, forming a thickened cap of epithelium around the distal segment of the NMN-complex. This experiment was repeated several dozen times with the same results.

In this experiment the epidermis manifests its capacity to shorten independently

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FIGURE 2. *Amaroucium constellatum;* right side of living larva. The epidermis (E), muscle cells (MC), notochordal cells (NC), notochordal lumen (I) and the tunic (T) are visible. Note the squamous epithelium within the notochord. Arrows indicate the position of the epidermis within the trunk.

FIGURE 3. *Amaroucium constellatum;* right side of same specimen as Figure 2, about one minute after the onset of tail resorption. Note the appearance of a space (ss) beneath the epidermis and the marked change in the arrangement and shape of the notochordal cells (NC). The epidermis (E) has separated from the surface of the muscle cells (MC) and has begun to thicken. The NMN-complex has begun the fold as a unit without a breakdown of the binding force or cementing substances between cells.

FIGURE 4. *Amaroucium constellatum;* right side of another specimen about two minutes after the onset of tail resorption. The NMN-complex has become more folded and has been partially forced into the posterior end of the trunk. The epidermis (E) has become thicker than it was in the larva.

FIGURE 5. *Amaroucium constellatum;* right side of same specimen as seen in Figure 4 about three minutes after the onset of metamorphosis. More than half of the tail is coiled within the posterior end of the trunk. The epidermis is greatly thickened.

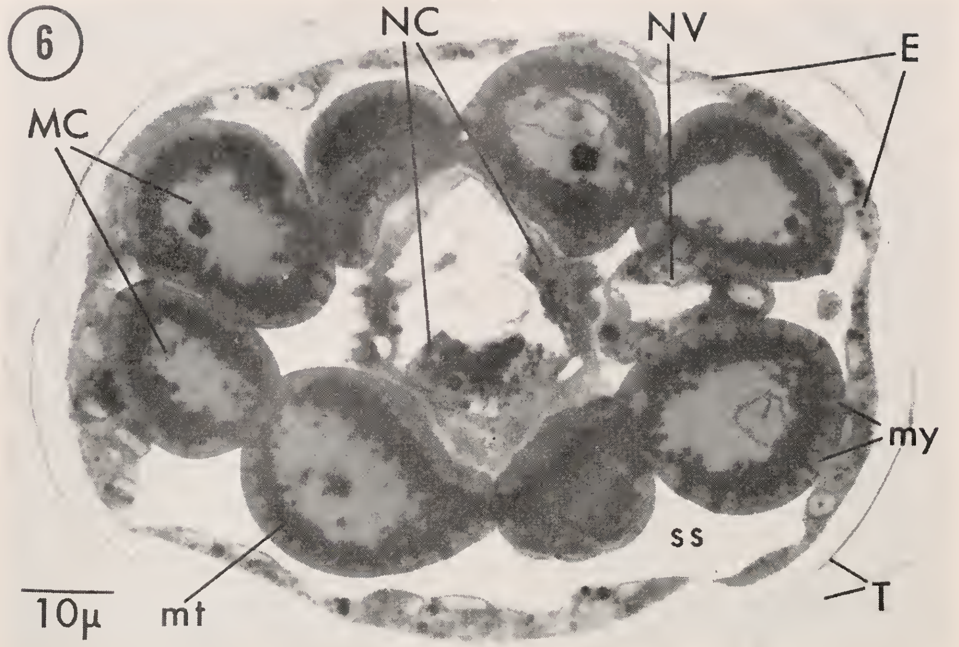


FIGURE 6. *Amaroucium constellatum.* Transverse section of the tail about two minutes after the onset of metamorphosis. A subepidermal space (ss) is prominent. The epidermis (E) is thicker than in the larva. Marked changes are apparent in the notochord. The cells (NC) tend to round up, filling the lumen. Many large vesicles are formed. The muscle cells (MC) increase somewhat in diameter as the NMN-complex bends and folds. One-micron Epon section; Richardson's stain.

of the other tissues. The NMN-complex is left bare by this action (except for the tunic which covers the entire organism). Under these circumstances the NMN-complex is never withdrawn into the trunk; the muscle cells do not manifest any capacity to shorten. These tissues remain unresorbed while the rest of the larva completes its metamorphosis.

Tails excised before the onset of metamorphoses do not undergo any of the characteristic histological changes observed in normal metamorphosing larvae or in tails excised after the onset of tail resorption. They twitch for many hours but eventually degenerate. The tail tissues are not necessary for post-larval development in *Amaroucium* (Scott, 1952), or in *Boltenia* (Cloney, 1961b). The caudal tissues have no known prospective significance but probably serve a nutritive function.

This rupturing of the caudal epidermis occurs spontaneously in some larvae collected in culture dishes. Numerous cases have been observed in which metamorphosis proceeded in the trunk without the resorption of the tail. This has also been reported by Scott (1952). Close inspection of more than a dozen of these specimens which failed to resorb their tails revealed that in all cases, there was a mass of epidermis at the base of the tail and at the tip of the tail, while the central portion of the tail was not covered by epidermal tissue.

Failure to resorb the tail may be attributed, at least in these cases, to the rupture of the epidermal envelope which normally contracts and is essential to the withdrawal of the NMN-complex.

The question of the energetics of contraction remains to be considered. The epidermis might shorten through elastic properties or it could actively contract and be dependent on aerobic oxidative processes for energy. The latter is suggested by the following experiment.

After the beginning of metamorphosis and following the onset of tail resorption, when the epidermis of the tail has separated from the underlying muscle, the larvae were placed for varying periods in a solution of 5×10^{-3} to 1×10^{-2} M KCN in sea water regulated to pH 8.0 with HCl (Fig. 11). Within about one minute after exposure to KCN the rate of shortening was slowed down. If the specimens were then washed in sea water, tail resorption would resume after a minute or two. Tail resorption is thus reversibly inhibited by potassium cyanide. Sodium azide has a similar inhibitory effect in a concentration of 10^{-2} M. Both of these substances have been reported to reversibly inhibit the *onset* of metamorphosis in *Amaroucium* larvae (Lynch, 1961). Potassium cyanide and sodium azide are well known for their inhibitory affects on cytochrome oxidase, the terminal enzyme complex in the respiratory chain (Wainio and Cooperstein, 1956; Pearse, 1960).

Discussion

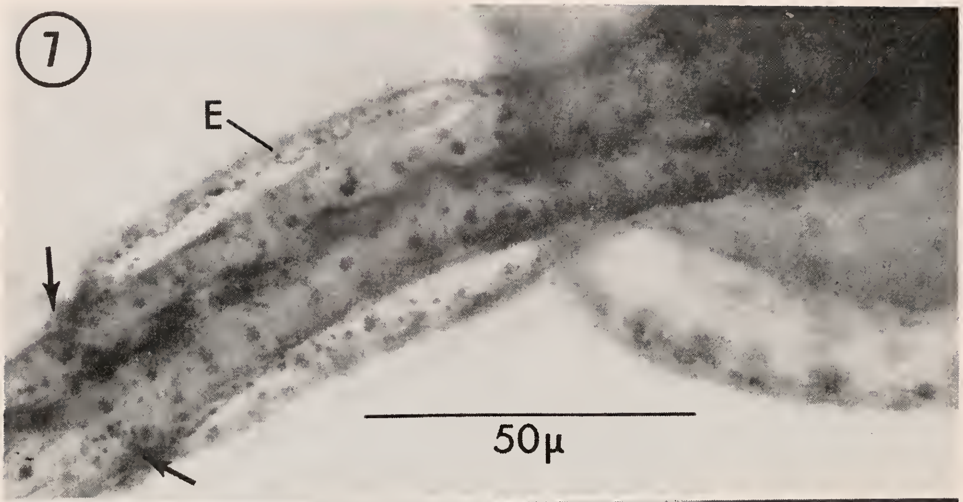
The overall aspects of tail resorption in *Amaroucium* correspond to Type 2 as mentioned in the introduction. This pattern of tail resorption has features in common with Type 1.

In both forms there are changes in the notochord. The notochordal matrix which contributes to the rigidity of the tail (hydrostatic skeleton) is released and leads to a loss of turgor and a partial collapse in both forms. This aspect of tail resorption was not reported by Weiss (1928) in his study of *Ciona*. In *Boltenia*, *Styela* and *Pyura*, both the muscle cells and notochordal cells become detached from the notochordal sheath as the tail is withdrawn. This process does not occur in *Amaroucium*, or other Type 2 forms. The muscle cells and notochordal cells remain associated in the same linear relationships, attached to the notochordal sheath. But in *Amaroucium* there is a rapid dissociation of the epidermis from the underlying muscle cells, and rapid changes in the notochordal cells.

The activity of a proteolytic enzyme might account for changes in the notochord and a breakdown of adhesion between tissues in all species. Conklin (1931) has made a similar suggestion to explain tail resorption in *Styela partita* but no direct evidence for the existence of a proteolytic or hydrolytic enzyme that is released or is activated at the time of metamorphosis has yet been found.

It seems reasonable, in view of the experiments with *Amaroucium* and the evidence of epidermal activities in *Ciona* (Weiss, 1928), and *Boltenia*, to generalize the statement that the contraction of the epidermis is responsible for forcing the tissues of the tail into the trunk at the time of tail resorption in ascidians.

Weiss' contention that tonic muscular contraction must contribute to the withdrawal of the tail seems untenable for the following reasons: (1) In Type 1 tail resorption the muscle cells become dissociated, and histological analyses show that the contractile elements become disarranged early in tail resorption. (2) In Type 2 tail resorption excision of the tail after the beginning of tail resorption leads to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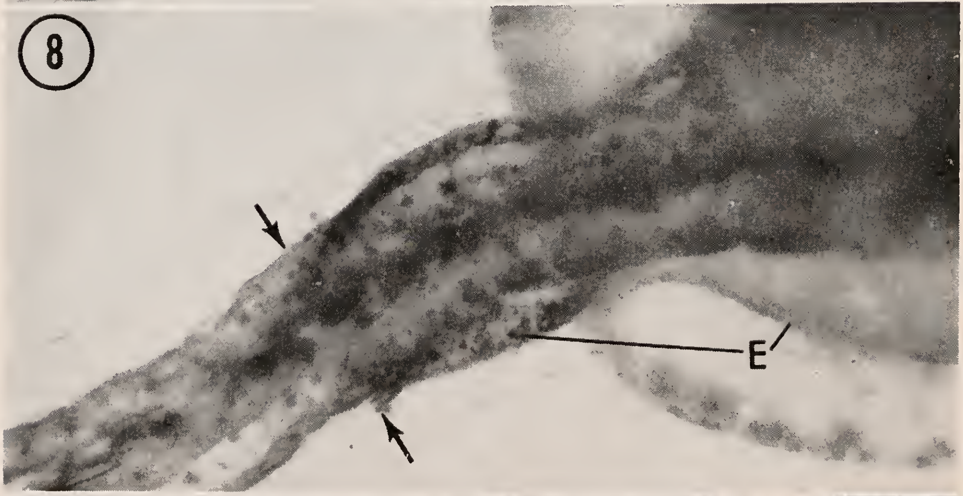
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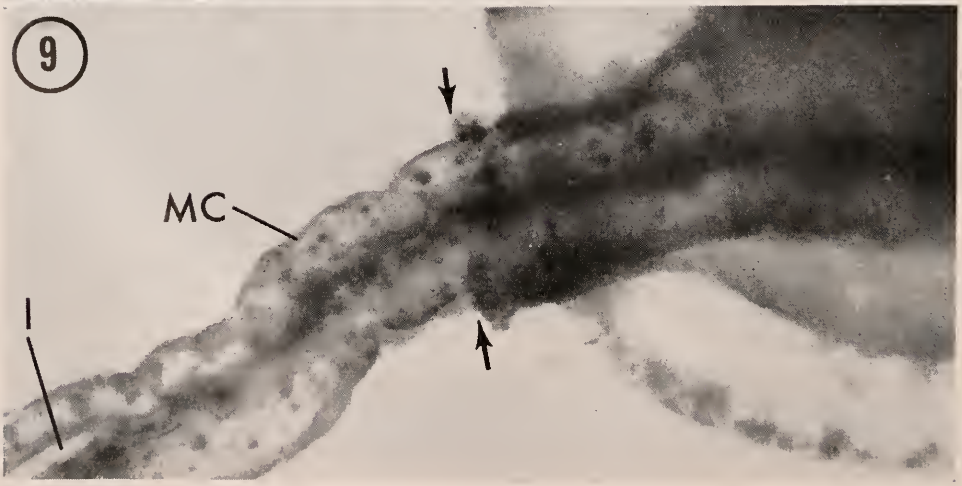
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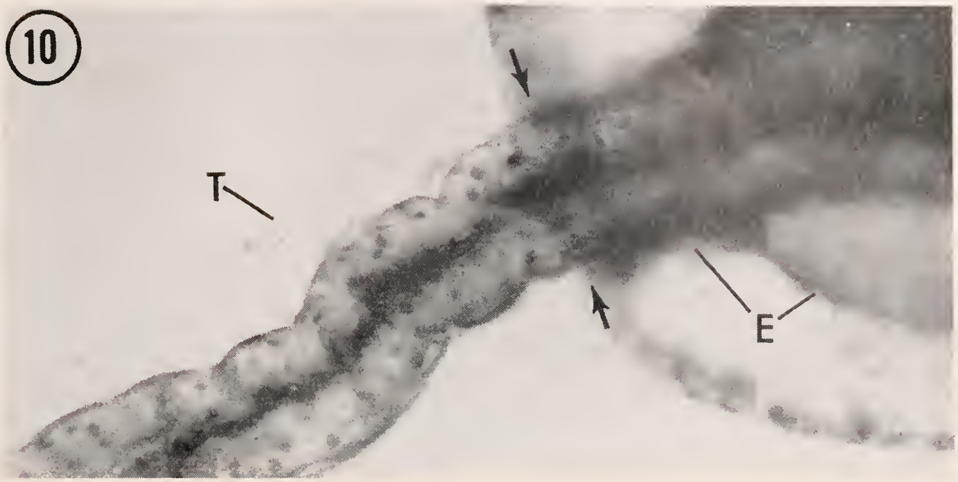

the retraction of the epidermis alone, while the NMN-complex actually pushes away from the trunk as if released from tension as soon as it detaches from the epidermis.

The mechanism of epidermal contraction is of general biological interest. The following is quoted from Hoffmann-Berling (1960, p. 346).

"All in all, it may be stated that systematic comparisons have not yet uncovered any dissimilarity which would indicate a fundamental difference between the contraction of a muscle and the contraction of an undifferentiated cell. The differences are only quantitative. The mechanism of muscle contraction is already present in the final form at the developmental stage of the single cell organism prior to the start of organ formation and tissue specialization. It is older than muscle itself."

Unpublished electron micrographs revealed filaments in the epidermis of *Amaroucium* larvae, but filaments are commonly found in epidermal cells, as well as many other epithelial cells in which active contraction is at least not obvious. Contractility may be one of the fundamental properties of all cells but in the case of tail resorption, some cells contract in a specific and predictable way while other cells of the tail do not perceptibly contract. These epithelial cells may be suitable subjects for further investigations of this long-standing problem.

SUMMARY

1. Tail resorption in *Amaroucium* is a rapid morphogenetic process. It is usually complete within only 6 minutes.

2. The initiation of tail resorption is signaled by a rapid separation of the epi-

FIGURES 7–10. *Amaroucium constellatum;* sequence of events following the excision of the distal one-third of the tail; living specimen. Immediately after the beginning of tail resorption the tail was excised and photographed. The epidermis began to shorten by contracting over the surface of the underlying NMN-complex. Within two minutes the epidermis shortened into an annular ring at the base of the tail. The NMN-complex invariably fails to be resorbed in this experiment. The epidermis of the distal segment (not shown) also shortens over the underlying tissues following excision. The arrows indicate the cut surface of the epidermis through stages of contraction.

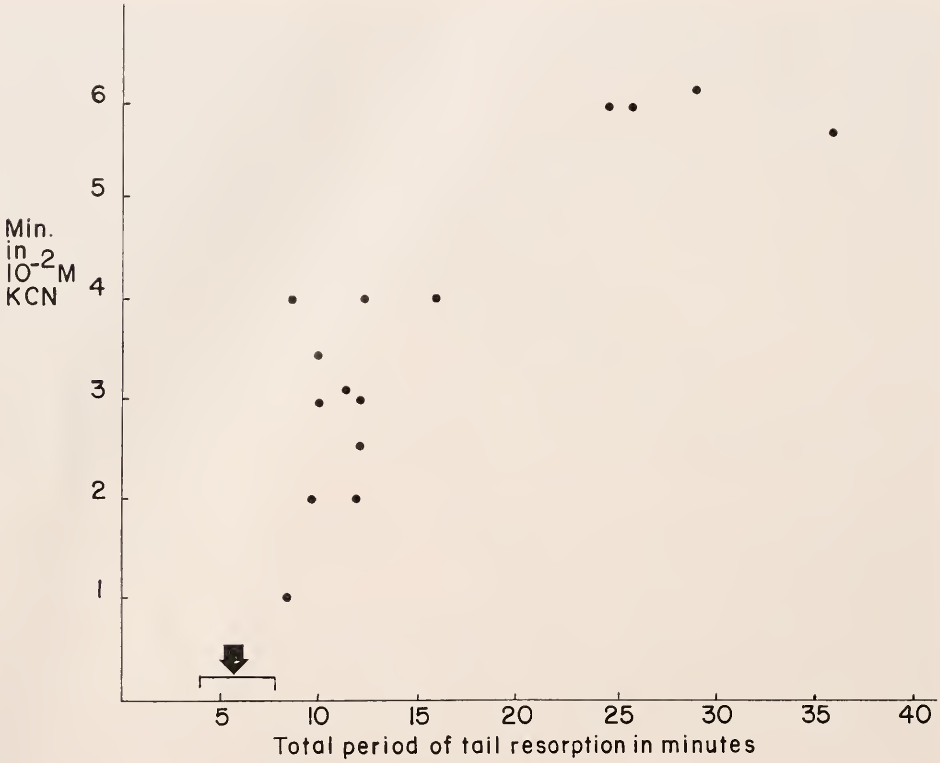

FIGURE 11. Inhibition of tail resorption by potassium cyanide. The total time required for the completion of tail resorption was plotted for larvae treated for various times in a solution of 10^{-2} *M* KCN. Experimental larvae were first observed until the first signs of tail resorption could be detected. They were then transferred to the KCN solution for from 1 to 6 minutes. They were subsequently washed in sea water. Cyanide treatment slows down the rate of tail resorption beyond the controls. The range of normal tail resorption is shown by a line on the lower left of the graph. The arrow indicates the average time of tail resorption in 14 normal specimens.

dermis of the tail from the underlying notochord-muscle-nerve cord complex (NMN-complex). This results in the formation of a fluid-filled subepidermal space. The NMN-complex buckles and folds as it moves into the posterior end of the trunk. The epidermis forms a thickened cap over the end of the trunk, enclosing the other tail tissue.

3. When the tail was excised after the beginning of tail resorption, the epidermis was observed to retract independently of the other tail tissues.

4. The muscle cells do not manifest any tendency to shorten without the epidermis.

5. Potassium cyanide and sodium azide reversibly inhibit the onset of metamorphosis and slow down the rate of tail resorption if they are applied after the beginning of metamorphosis.

6. Some histological changes in the tail of *Amaroucium* and other species of ascidians may be the result of the activity of a proteolytic enzyme.

LITERATURE CITED

Berrill, N. J., 1947. Metamorphosis in ascidians. *J. Morph.*, **81**: 249–267.

Cloney, R. A., 1961a. Observations on the mechanism of tail resorption in ascidians. *Amer. Zool.*, **1**: 67–87.

Cloney, R. A., 1961b. Changes in the fine structure of striated muscle cells during metamorphosis in ascidian larvae. *Anat. Rec.*, **139**: 217–218.

Conklin, E. G., 1931. The development of centrifuged eggs of ascidians. *J. Exp. Zool.*, **60**: 1–119.

Grave, Caswell, 1921. *Amaroucium constellatum* (Verrill) II. The structure and organization of the tadpole. *J. Morph.*, **36**: 71–91.

Grave, Caswell, 1935. Metamorphosis of ascidian larvae. Papers from the Tortugas Lab. *Carn. Inst. Wash. Publ., No.* **452**: 209–292.

Hoffmann-Berling, Hartmut, 1960. Other mechanisms producing movements. *In:* Comparative Biochemistry, Marcel Florkin and Howard S. Mason, editors. Vol. 2, pp. 341–370. Academic Press, New York.

Jackson, Sylvia Fitton, 1958. Some aspects of morphogenesis in ascidians. *Biol. Bull.*, **115**: 335.

Lynch, W. F., 1961. Extrinsic factors influencing metamorphosis in bryozoan and ascidian larvae. *Amer. Zool.*, **1**: 59–66.

Luft, John, 1961. Improvements in epoxy resin embedding methods. *J. Cell Biol.*, **9**: 409–414.

Pearse, A. G. E., 1960. Histochemistry. Little, Brown and Co., Boston.

Richardson, K. C., L. Jarett and E. H. Finke, 1960. Embedding in epoxy resins for ultrathin sectioning in electron microscopy. *Stain Tech.*, **35**: 313–323.

Scott, Sister F. M., 1946. The developmental history of *Amaroecium constellatum*. II. Organogenesis of the larval action system. *Biol. Bull.*, **91**: 66–87.

Scott, Sister F. M., 1952. The developmental history of *Amaroecium constellatum*. III. Metamorphosis. *Biol. Bull.*, **103**: 226–241.

Wainio, W. W., and S. J. Cooperstein, 1956. Some controversial aspects of mammalian cytochromes. *Adv. in Enzym.*, **17**: 329–392.

Weiss, Paul, 1928. Experimentelle Untersuchungen über die Metamorphoses der Ascidien II. Versuche über der Mechanismus der Schwanzinvolution. *Biol. Zentr.*, **48**: 387–407.

MOLTING AND CYCLIC ACTIVITY IN CHROMATOPHOROTROPINS OF THE CENTRAL NERVOUS SYSTEM OF THE BARNACLE, BALANUS EBURNEUS [1]

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Although molting in the acorn barnacles is more frequent than that described for most Crustacea, every 2–3 days in the warm-water species, and continues throughout the life of the individual barnacle (Costlow and Bookhout, 1953, 1956), nothing is known of the mechanisms which control the sequence of molting or the duration of the intermolt period (Carlisle and Knowles, 1959). Barnes and Gonor (1958a) have suggested that the control of molting in the Cirripedia may depend on the absence of a molt-inhibiting hormone and thus be more similar to the mechanism described for *Lysmata seticaudata* (Carlisle, 1953a, 1953b, 1953c; Carlisle and Dohrn, 1953) than the mechanism described for most decapods which is thought to involve the interaction of a molt-accelerating and a molt-inhibiting hormone (Passano, 1960, 1961).

Several attempts have been made to establish a relationship between cyclic activity of barnacle shell and body tissues and the regular sequence of molting periods. While cyclic activity has been described from a few tissues in barnacles (Thomas, 1944; Costlow, 1956), the activity has not been shown to be directly associated with either ecdysis or its control.

The present study has had two major objectives: (1) to determine if the *Uca* black-pigment-dispersing substance of the central nervous system of acorn barnacles (Sandeen and Costlow, 1961) is cyclic in its activity; and (2) if activity of this chromatophorotropin is cyclic, to determine whether the changes in activity are associated with the molting cycle of the adult barnacle.

METHODS

Adults of *Balanus eburneus* were collected from pilings in the area of the Duke University Marine Laboratory and placed in the running sea water system. When it had been determined that the animal had not been damaged in the course of removal from the pilings, the barnacles were segregated into plastic compartmented boxes, placed at a slight angle under the direct flow of the sea water outlet, and fed *Artemia* nauplii. The barnacles were checked at regular half-hour intervals during the day and if a molt were found, the time was recorded and the animal removed to another container in running sea water.

At the intervals of time after molting shown in Table I the barnacle body was removed from the shell, dried on filter paper, and weighed to the nearest 0.1 mg. on a Roller-Smith balance. The central nervous system was then dissected intact

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[1] These studies were aided by a contract, 104-194, between the Office of Naval Research and Duke University.

TABLE I

Number of adult Balanus eburneus from which central nervous systems were extracted at intervals following molting and the dilutions of extract (CNS/ml) used

	Intervals Hours after molting					
Dilution	0	12	24	48	72	96
1:1	12	12	12	11	8	9
1:5	19	—	20	10	9	9
1:10	9	—	9	—	—	—

from the body and thoroughly triturated in a glass dish containing as little water as possible. The ground material was suspended in the desired amount of filtered sea water, boiled, and centrifuged.

Fiddler crabs, *Uca pugilator*, which had been destalked 12 to 24 hours previously, were then injected with the barnacle central nervous system extract. The standard dose was 0.05 cc., injected into the ventral hemocoel at the base of the fifth walking leg with a tuberculin syringe and a 26-gauge needle. Three different concentrations of extract were used: one central nervous system per one ml. of sea water, one per five ml. and one per ten ml. For control injections, homogenized opercular muscle from the barnacles was used and 5 fiddler crabs were injected daily with *Uca* eyestalk extract, one pair per 5 ml., to determine the variability of the bioassay animals as well as the technique.

Five eyestalkless fiddler crabs were injected with the extract from each central nervous system and the chromatophores of each animal staged at intervals of 15, 30, 45, 60, 90, 120, and 180 minutes following injection. The chromatophore scale of Hogben and Slome (1931) was used to determine the *Uca* chromatophore response to the barnacle central nervous system extracts. From the average chromatophore values for each reading the total net activity was determined for the entire 180-minute period (Sandeen and Costlow, 1961). All experiments were begun between 1 and 2 PM and terminated before 5 PM to avoid possible fluctuations in the crab chromatophores due to diurnal rhythms.

RESULTS

Figure 1 gives the average total activity values for extracts of *B. eburneus* central nervous systems removed at regular intervals following ecdysis and assayed on eyestalkless fiddler crabs. At a concentration of 1:1 the central nervous system had a low activity immediately following molting, followed by an increase in activity at 24 hours after molting. Statistical treatment of the data from these experiments indicates that variability in activity of the extracts within the sample is as high as that between the samples, and that the fluctuations in central nervous system activity at different periods of time after ecdysis are not significant at a concentration of 1:1.

Injections of the barnacle central nervous system extract at a dilution of 1:5 showed similar changes in the activity of the chromatophorotropins in relation to the molting cycle: a low point of activity immediately following molting, a peak

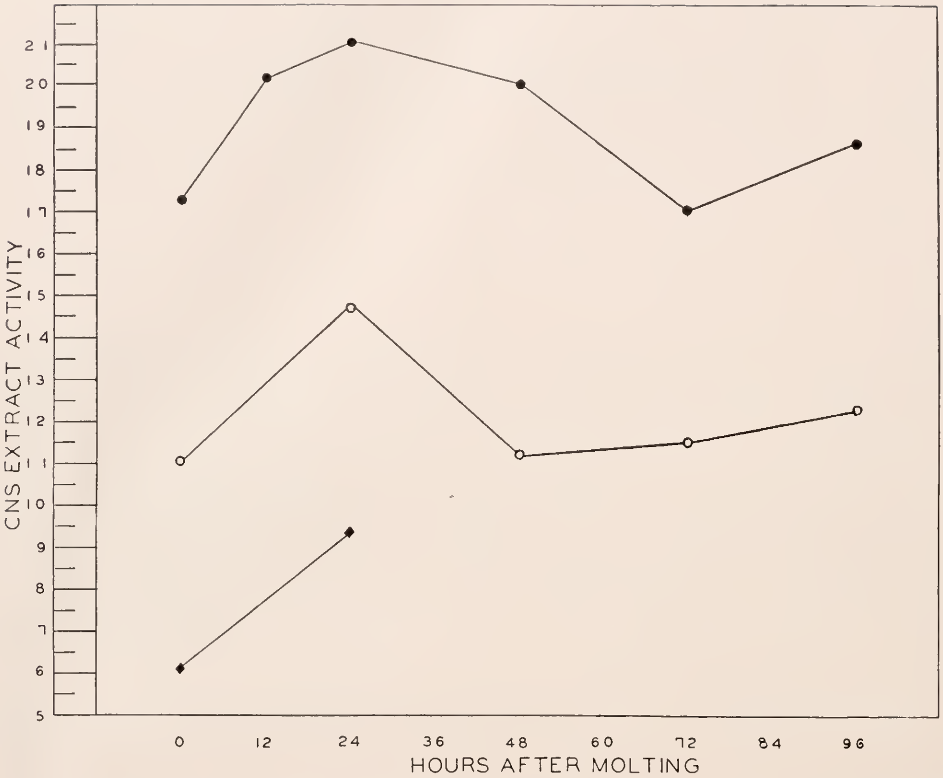

FIGURE 1. Average activity values of *Balanus eburneus* central nervous systems extracted at intervals following ecdysis and analyzed on eyestalkless fiddler crabs, *Uca pugilator:* solid circles, 1:1 dilution; open circles, 1:5 dilution; and solid diamonds, 1:10 dilution.

of activity at 24 hours after molting, followed by a decline in activity to the initial low point, and a slight increase in activity at 72 and 96 hours after molting. These data, when subjected to an analysis of variance, are highly significant at the 95% level.

Dilutions of the barnacle central nervous system extract of 1:10 also showed an activity which was low at the time of molting. This was followed by an increase at 24 hours after molting but insufficient data were available for additional time intervals to make further comparisons. Table II gives the total activity values for all dilutions of extracts used in the experiment.

Injection of barnacle opercular muscle extracts did not produce any reaction in the eyestalkless fiddler crab chromatophores. Day-to-day fluctuations in the chromatophore activity of the *Uca* controls (*Uca* injected with extracts of *Uca* eyestalks) were slight and never of sufficient magnitude to affect significantly the activity values obtained for the barnacle extracts.

Groups of barnacles from which central nervous systems were assayed represented a range of body weights from 24 mg. to 160 mg. Although this does not exceed a factor of 10 it was considered desirable to be sure that any consistent

TABLE II

Total activity values of central nervous systems of Balanus eburneus extracted at intervals within one molting period

	Hours after molting					
	0	12	24	48	72	96
1:1 Dilution	9.2	22.4	24.8	24.4	18.8	20.6
	21.8	22.6	23.8	23.6	12.4	20.8
	11.2	19.6	24.7	22.4	19.4	21.8
	19.8	17.8	9.6	24.0	22.4	15.0
	21.6	18.4	22.8	20.4	15.2	22.0
	20.2	20.2	18.6	12.8	11.2	9.0
	19.4	18.4	20.8	19.8	16.4	18.0
	11.2	24.0	20.0	11.6	20.4	21.6
	20.6	21.4	21.8	18.6		19.6
	11.4	20.4	17.0	23.4		
	21.3	14.6	24.8	20.2		
	19.6	22.0	24.4			
Average	17.3	20.2	21.1	20.1	17.0	18.7
1:5 Dilution	5.8		16.0	5.4	11.0	13.4
	14.6		15.2	13.0	9.6	13.0
	8.2		14.0	15.4	11.2	13.4
	7.2		11.0	10.4	9.8	12.6
	9.0		12.4	10.2	8.6	10.0
	9.6		14.4	9.8	14.8	12.6
	7.4		14.2	13.2	10.6	12.2
	11.3		17.6	13.4	11.8	12.4
	8.0		11.4	7.6	16.0	11.6
	14.8		14.3	14.0		
	13.5		18.2			
	12.3		6.0			
	18.4		10.8			
	9.6		18.2			
	12.8		17.8			
	16.4		18.8			
	13.2		17.6			
	11.4		15.6			
	3.6		16.4			
Average	11.0		15.5	11.2	11.5	12.4
1:10 Dilution	6.8		11.2			
	10.6		2.4			
	9.0		3.8			
	3.8		10.2			
	6.4		9.6			
	9.4		14.8			
	6.6		11.0			
	2.3		8.6			
	0.0		12.8			
Average	6.1		9.4			

change in the level of central nervous system activity was not solely a function of body size or weight. Accordingly, the data for central nervous system activity and the corresponding barnacle body weights were analyzed. The statistical treatment of these data indicates that body weight and activity of the chromatophorotropins within the central nervous system are independent.

DISCUSSION

Although ecdysis in the barnacles, or Cirripedia, is analogous to the process described in considerable detail for the Decapoda, there is little evidence to date that the control of molting or the mechanisms of control in these two groups of Crustacea are similar. Attempts have been made to compare molting in the Cirripedia and in the Brachyura, either by studying possible sites of endocrine activity or storage organs or through investigations of physiological responses which may be similar. Most of our knowledge of the control of molting or the mechanisms of control in barnacles, however, is restricted to that gleaned from negative results.

Costlow and Bookhout (1958), studying the relationship between metabolic rate and the molting cycle in *Balanus amphitrite*, found that while variations in oxygen consumption did occur within any one molting period, there was no indication that an increase in respiratory rate occurred prior to molting, comparable to the two-fold increase described for some decapods (Roberts, 1957a, 1957b). Crisp and Patel (1960) described an endogenously regulated anecdysis in *Balanus balanoides* but Barnes (1962), working with the same species of barnacle, has suggested that the molting rhythm of this species is determined to a greater extent by environmental factors than endogenous factors. While temperature, diet, abundance of food, and reproductive state have been shown to affect the frequency of molting, at least one environmental factor which affects molting in some decapods, photoperiod, does not influence the molting frequency of barnacles (Costlow and Bookhout, 1956).

Evidence for a series of cyclic cellular changes within each molting period, comparable to those described for decapods by Drach (1939), has been scant. Thomas (1944) described the cyclic activity of the sublingual and subesophageal glands in *Balanus perforatus* and, while the cycles were concurrent, there was no indication that the activity of the glands was even indirectly involved in the control of molting. Costlow (1956) reported a secretory cycle in the shell-forming tissues in *Balanus improvisus* but cellular activity, as well as subsequent shell growth, was not associated with the 2–3-day molting frequency. Barnes and Gonor (1958a, 1958b) found neurosecretory cells in the central nervous system of several species of barnacles and gave a detailed description of the types of cells in *Pollicipes polymerus*, but did not associate the cyclic activity of these cells with the molting cycle. They also studied sections of whole bodies of *P. polymerus* but were unable to locate any storage organs for neurosecretory material and considered the possibility that discharge of such products may occur directly from the cells into the perineural blood sinus.

Sandeen and Costlow (1961) described one basic similarity which does exist between the Cirripedia and the Decapoda. They found two chromatophorotropins in the central nervous system of *Balanus eburneus*, *Chelonobia patula*, and *Lepas* sp. which were similar in activity to the *Uca* black-dispersing substance and the *Palaemonetes* red-pigment-dispersing material. The function of these activators, especially in the absence of either chromatophores or eyestalks, or their possible role in molting of barnacles was not investigated, however.

In the present study it has been established that activity of the one chromatophorotropin within the central nervous system of *B. eburneus*, the *Uca* black-dispersing substance, is cyclic and that the cycle does conform to that demonstrated for the two–three-day molting cycle. The greatest changes in level of activity are those at the time of molting and at 48 hours after molting, when the activity is lowest, and at 24 hours after molting when the activity is highest (Fig. 1). The slight increases in activity at 72 and 96 hours after molting are not statistically significant but do deserve some comment. Normally, at the temperatures under which the experimental barnacles were maintained (23°–26° C.), the frequency of molting is every 2–3 days. Thus, at 72 hours after the initial molt the barnacle would normally have begun the next molt and the activity of the chromatophorotropin would be low (Fig. 1). At 96 hours after the first molt the animal would normally have completed the next molt and the activity might be expected to equal the peak which corresponds to that shown for the 24-hour interval following ecdysis. In animals which do not initiate the mole or complete it within the regular 2–3-days frequency the activity of the central nervous system extracts never attained the peak described for the 24-hour interval following molting.

While there is no evidence that the chromatophorotropins of the central nervous system of barnacles are directly associated with the control of molting, the present study does suggest several possible functions and relationships. If we accept the suggestion of Barnes and Gonor (1958b) that in the absence of a storage site the neurosecretory products of the central nervous system are released directly into the hemolymph, it is possible to carry the concept of hormonal control of molting in the barnacles one step further. In the absence of a storage site the neurosecretory products are accumulated in the central nervous system itself. Secretory activity of the cells would increase following molting and reach a peak at 24 hours following ecdysis, the point of highest activity and presumably the highest concentration. This would be followed by a gradual release of the material into the hemolymph during the next 24-hour period until the initial low level was again reached in the central nervous system. If these products were to include a molt-accelerating substance, the release of material into the hemolymph between 24 and 48 hours following molting would serve to stimulate those additional physiological processes which are prerequisite to the actual process of molting. Following ecdysis the cycle would be repeated, continuing the 2–3-day frequency of molting.

In barnacles which have not molted for a second time within 96 hours following the previous molt, the absence of an increase in activity of the chromatophorotropins of the central nervous system, comparable to that found at 24 hours after molting, can be attributed to at least one of two things: (1) secretory activity within the central nervous system may have been insufficient to accumulate products equal to the titer found during the previous interval and thus molting is not initiated, or (2) the general physiological level of the animal, or the general metabolic state, had been reduced by environmental factors in the laboratory, and all functions, including molting, were retarded. Because the central nervous system extract of any one barnacle can only be analyzed for one time interval within any one molt, it is not possible to follow the entire cycle of activity of the chromatophorotropins within any one barnacle.

Further studies obviously are needed to delineate the various components of the central nervous system extracts and determine, either by injection or implantation, if any of the various fractions can affect molting in the Cirripedia or the Brachyura.

SUMMARY AND CONCLUSIONS

1. The central nervous system of the barnacle, *Balanus eburneus,* was removed at known intervals following ecdysis, extracted in sea water, and assayed by injecting into eyestalkless *Uca pugilator* to determine if the barnacle chromatophorotropins exhibited a cyclic activity associated with molting.

2. A cyclic pattern of activity was observed within one intermolt period. The changes in activity of the *Uca* black-pigment-dispersing substance, highly significant at the 95% level, were the low concentrations immediately following molting and 48 hours after ecdysis and the high concentration which occurred at 24 hours after molting. In barnacles which did not molt again within the usual 72–96-hour period following the first molt, the level of activity of the central nervous system extracts remained low. Body weight and the activity of the central nervous system extracts were found to be independent.

3. The hypothesis is presented that in the absence of a storage organ comparable to the sinus gland of Brachyura, neurosecretory products originating from the central nervous system are released directly into the blood. The release of these products following the period of greatest concentration, 24 hours after molting, stimulates the physiological processes and cellular changes which culminate in ecdysis.

LITERATURE CITED

BARNES, H., 1962. So-called anecdysis in *Balanus balanoides* and the effect of breeding upon the growth of the calcareous shell of some common barnacles. *Limnol. Oceanogr.,* **7**: 462–473.

BARNES, H., AND J. J. GONOR, 1958a. Neurosecretory cells in the Cirripede, *Pollicipes polymerus* J. B. Sowerby. *J. Mar. Res.,* **17**: 81–102.

BARNES, H., AND J. J. GONOR, 1958b. Neurosecretory cells in some cirripedes. *Nature,* **181**: 194.

CARLISLE, D. B., 1953a. Studies on *Lysmata seticaudata* Risso (Crustacea Decapoda). III. On the activity of the moult-accelerating principle when administered by the oral route. *Pubbl. Staz. Zool. Napoli,* **24**: 279–285.

CARLISLE, D. B., 1953b. Studies on *Lysmata seticaudata* Risso (Crustacea Decapoda). IV. On the site of origin of the moult-accelerating principle—experimental evidence. *Pubbl. Staz. Zool. Napoli,* **24**: 285–292.

CARLISLE, D. B., 1953c. Studies on *Lysmata seticaudata* Risso (Crustacea Decapoda). VI. Notes on the structure of the neurosecretory system of the eyestalk. *Pubbl. Staz. Zool., Napoli,* **24**: 435–447.

CARLISLE, D. B., AND P. F. R. DOHRN, 1953. Studies on *Lysmata seticaudata* Risso (Crustacea, Decapoda). II. Experimental evidence for a growth and moult-accelerating factor obtainable from eyestalks. *Pubbl. Staz. Zool., Napoli,* **24**: 69–83.

CARLISLE, D. B., AND F. KNOWLES, 1959. Endocrine Control in Crustaceans. Cambridge University Press.

COSTLOW, J. D., JR., 1956. Shell development in *Balanus improvisus* Darwin. J. Morph., **99**: 359–415.

Costlow, J. D., Jr., and C. G. Bookhout, 1953. Moulting and growth in *Balanus improvisus*. *Biol. Bull.*, **105**: 420–433.

Costlow, J. D., Jr., and C. G. Bookhout, 1956. Molting and shell growth in *Balanus amphitrite niveus*. *Biol. Bull.*, **110**: 107–116.

Costlow, J. D., Jr., and C. G. Bookhout, 1958. Molting and respiration in *Balanus amphitrite* var. *denticulata* Broch. *Physiol. Zoöl.*, **31**: 272–280.

Crisp, D. J., and B. S. Patel, 1960. The moulting cycle in *Balanus balanoides* L. *Biol. Bull.*, **118**: 31–47.

Drach, P., 1939. Mue et cycle d'intermue chez les Crustacés décapodes. *Ann. Inst. Oceanogr., Monaco*, **19**: 103–391.

Hogben, L. T., and D. Slome, 1931. The pigmentary effector system. VI. The dual character of endocrine coordination in amphibian colour change. *Proc. Roy. Soc. London, Ser. B*, **108**: 10–53.

Passano, L. M., 1960. Molting and its control. *In:* The Physiology of Crustacea, vol. 1: pp. 473–536. Academic Press, New York and London.

Passano, L. M., 1961. The regulation of crustacean metamorphosis. *Amer. Zool.*, **1**: 89–95.

Roberts, J., 1957a. Thermal acclimation of metabolism in the crab, *Pachygrapsus crassipes* Randall. I. The influence of body size, starvation, and molting. *Physiol. Zoöl.*, **30**: 232–242.

Roberts, J., 1957b. Thermal acclimation of metabolism in the crab *Pachygrapsus crassipes* Randall. II. Mechanisms and the influence of season and latitude. *Physiol. Zoöl.*, **30**: 243–255.

Sandeen, M. I., and J. D. Costlow, Jr., 1961. The presence of decapod-pigment activating substances in the central nervous system of representative Cirripedia. *Biol. Bull.*, **120**: 192–205.

Thomas, H. S., 1944. Tegumental glands in Cirripedia Thoracica. *Quart. J. Micr. Sci.*, **84**: 257–282.

PROPRIOCEPTION IN THE LEGS OF PHALANGIDS

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The presence of thin areas in the exoskeleton of certain arachnids and other arthropods has been known for about 80 years. The thin areas, made up of epicuticle, are usually bordered by a thickened lamella and occur in two general shapes—slit-like and circular. Isolated, slit-like thin areas were noted by Bertkau (1878), while groups of more or less parallel slits resembled the ancient stringed instrument, the lyre, and prompted the term, "lyriform organs" (Gaubert, 1890). The circular ones were first described by Berlese (1909) and called "campaniform sensillae." Typically, the dendritic process of a bipolar neuron is attached to the epicuticle or to the bordering lamella. Snodgrass (1935) and Kaston (1935) have summarized the histology of the former condition in insects. In 1938 Pringle presented evidence from the cockroach that indicated stress reception as the real function.

Recently, the attachment of the neurite to the bordering lamella has been described with electron micrographs by Salpeter and Walcott (1960), and shown to function as a vibration receptor in spiders. The neurite is stimulated by stretching in the region of attachment to the epicuticle. Most commonly this is done by compression pressure on the bordering lamella. This results in exaggerating the convexity of the epicuticle.

These organs exhibit considerable morphological variation and have been reported in insects, spiders, scorpions, mites, ticks and phalangids (Gaubert, 1892; Hansen, 1893; Hansen and Sorensen, 1904). The present paper illustrates campaniform and slit organs (isolated, grouped, and lyriform) of phalangids and reports evidence suggesting the function of at least the campaniform organs to be proprioceptive.

Procedure and Results

Legs of phalangids possess several shapes and combinations of sensillar organs. The location and number on each of the four legs for six species are indicated in Table I. Considerable similarity exists among all of the species shown except *Caddo agilis*. In addition to the sensillar arrangement, this form is distinguished from the others in Table I by size, habitat and morphology.

Data for all the leg segments are included except for the coxa. On this segment, typically a single slit sensillium occurs on the distal margin. At the distal margin of the trochanter there occurs, except in *Caddo*, a lyriform organ with a fairly constant number of slits—seven, for example, in *Phalangium opilio* (Fig. 1). Small clusters of I- and L-shaped slit sensillae occur on the proximal portion of the femur (Figs. 1 and 2). The leg autotomy plane is located between the trochanter and femur. Presumably, the rich sensillar supply on these segments functions to indicate to the animal mechanical stress upon this plane. Large, single slits are

TABLE I

Location and number of proprioceptor organs on the legs of certain phalangids

Leg segment	*Caddo agilis* Leg number				*Opilio parietinus* Leg number				*Phalangium opilio* Leg number			
	1	2	3	4	1	2	3	4	1	2	3	4
Trochanter	0	0	0	0	4	2	4	5	7	7	7	7
Femur—P*	9	9	9	5	18	16	22	21	29	30	30	29
—S*	1	1	1	1	2	4	2	2	4	5	4	4
—D*	1	1	1	1	1	1	1	1	1	1	1	1
Tibia—P*	2	?	2	2	6	3	6	6	9	8	8	9
Metatar. P*					7	5	7	7	9	9	8	9
Tarsus 1st*						1	1		1 (2)	1 (2)	1	1 (2)
Mid.*							6	6	7–12	11–14	9–10	7–11

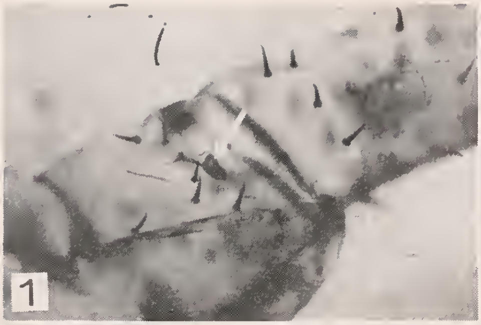
Leg segment	*Leiobunum calcar* Leg number				*Leiobunum longipes* Leg number				*Leiobunum politum* Leg number			
	1	2	3	4	1	2	3	4	1	2	3	4
Trochanter	5	5	5	5	6	7	7	5	5	5	4	6
Femur—P*	21	24	23	24	30	31	30	27	23	27	22	29
—S*	4	5	4	5	4	5	4	4	3	5	4	4
—D*	1	1	1	1	1	1	1	1	1	1	1	1
Tibia—P*	7	5	5	7	7	7	7	6	7	7	7	9
Metatar. P*	8	6	7	8	8	8	8	8	8	10	8	10
Tarsus 1st*	1	1	1	1	1	0	1	1–2	1	1	1	1
Mid.*	7	7	8	6–8	8–11	13–16	6–10	10–11	4	9	6	7–8

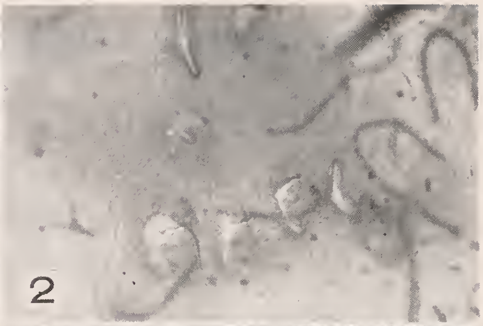
* Key to symbols:

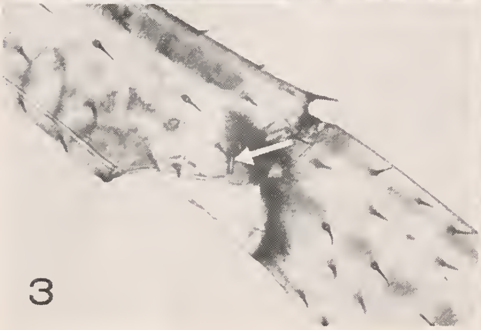
P—organs located on proximal portion of leg segment.
S—shank of leg segment.
D—distal portion of leg segment.
1st—number of organs found on the most proximal article of tarsus.
Mid.—the number of the article on which is found a campaniform organ. Extremes (for example, 7–12) indicate that organs have been found on articles within these limits.

more or less evenly spaced along the shaft of the femur. The largest slit organ on the leg occurs on the distal femur where it articulates with the patella (Fig. 3). The ventral, proximal portion of the tibia has a cluster of campaniform sensillae. Usually, three slit organs and three to seven campaniform sensillae occur on the dorsal surface of the metatarsus near the articulation with the tibia. Occasionally one or two of these latter organs are separated and distal from the cluster. Typically, two solitary campaniform organs are found on the tarsus; one is on the proximal-most article and the other on an article in the middle one-third of this segment (Fig. 4). Observations of the organs tabulated in Table I were made from exuviae of the final molt.

Although Savory (1962) and others have indicated that the second pair of legs possess special senses, the arrangement and kinds of sensillar organs listed in Table I do not show any consistent disparity with the other three pairs. Legs from all four pairs were used in the experimentation; however, no differences be-







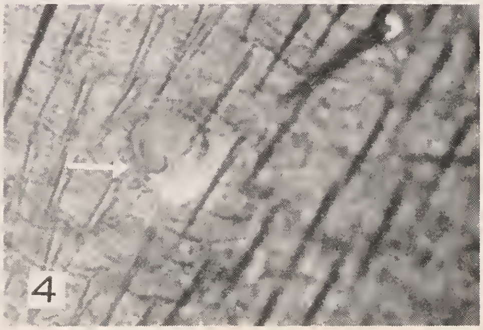


FIGURE 1. Lyriform organ at distal margin of trochanter (arrow). The autotomy plane which separates trochanter from femur is shown under the other end of the arrow. A cluster of I- and L-shaped slit organs may be seen on the proximal portion of the femur (right).

FIGURE 2. Several slit organs clustered at base of femur (see Figure 1). The expanded area in the middle of the slit probably receives the attachment of a bipolar neuron.

FIGURE 3. Large, thick-bordered slit organ on femur (arrow) where femur articulates with patella.

FIGURE 4. Campaniform sensillium on twelfth article of the tarsus; the structure measures approximately 20 by 15 microns. Figures 1–4 are photographs made from molt cases of *Phalangium opilio* L.

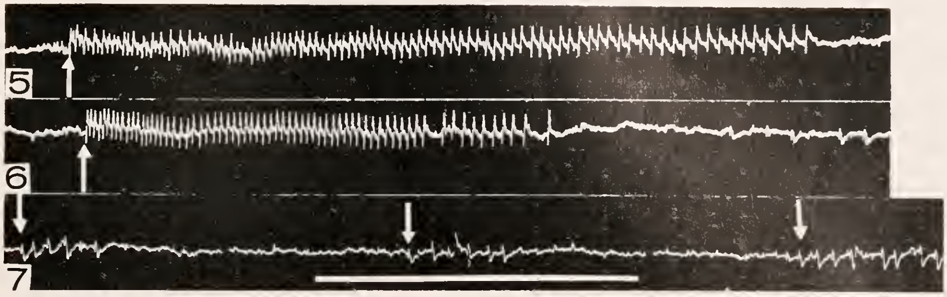
tween legs were observed in the records obtained. Oscilloscope traces shown in Figures 5–7 were obtained from the left leg I of *Phalangium opilio*.

Legs from healthy adults were removed at the autotomy plane between the trochanter and femur and mounted upon a Plasticene support. Platinum wire electrodes inserted into the open, proximal end and the patellar end of the femur were connected via a Grass Type P. 4 preamplifier to a Tektronix 531 oscilloscope.

By adequate support of all sections of the leg except the test area, stress could be placed on specific sensillae. Stress was produced by hyperextending or hyperflexing the normal angle of movement at an articulation nearest the sense organ. This was done by manually-operated glass and steel probes.

Although pressure was applied on all joints of the leg, action potentials were consistently obtained only upon hyperextension of the tibiometatarsal joint (Fig. 5) and hyperflexion of the tarsometatarsal joint (Fig. 6) and tarsal articles. Adaptation was rapid and complete.

To localize the site of origin of the nervous activity shown in Figures 5 and 6,



Figures 5 and 6. Oscilloscope records from leg nerve at the femur. Figure 5. Forced extension of the tibiometatarsal joint of *P. opilio* L. Figure 6. Forced flexion of a tarsometatarsal joint of the same species. Arrows indicate time at which stress was applied.

Figure 7. Three oscilloscope records of extension of tarsometatarsal joint. The typical pattern of this trace may be seen in Figure 6 near the right-hand side. Arrows indicate the moment of flexing; the time bar shows one-second interval on Figures 5, 6 and 7.

articles of the tarsus and metatarsus were removed, a few at a time, by cutting. Impulses continued to be elicited, upon stress, in a normal manner until the immediate region of a sensillium was removed; then the record ceased. Local destruction attempts were unsuccessful.

A series of differently-shaped impulse discharges usually appeared on the record about 0.4 second after adaptation (Fig. 6). It was interesting to note that a wave form which had a similar shape appeared upon moving the leg segment in the direction opposite to that producing the stress discharges and without exceeding the normal limits of movement. Figure 7 indicates three such records from extension of the tarsometatarsal. Just where this second discharge originated is unknown, possibly in a different sense organ located nearby, or perhaps two neurons are associated with the same sensillium to transmit "action" and "reaction" stimuli. However, morphological descriptions known to the writer have shown only one nerve cell involved for each organ.

Attempts to induce distinct responses from the grouped slits (Figs. 1 and 2) generally failed. However, surface pressure from careful scraping of the area with glass and steel probes resulted in a jumble of electrical activity. When nearby spines were intentionally prodded, no response was obtained. This was sufficient to suggest that impulses were coming from the slit organs. These manipulations were observed plainly under suitable magnification.

The slit sensillae of the femur, with one exception, have yielded no clear response to stimulation. On this one occasion it is believed that insertion of the electrode into the femoropatellar junction caused pressure to activate the large single slit sensillium at that joint (Fig. 3). Very regular, large, fast impulses continued for many minutes. They presumably did not originate from leg segments other than the femur since manipulation of these segments caused no alteration in the frequency or amplitude of these discharges. Subsequent repositioning of the femoropatellar electrode abolished the response. It is possible that one of the other sensillae located on the shank of the femur was the organ responding. This is unlikely, however, in that lateral movements of the shank, designed to place stress upon these organs, produced no response.

Response to parameters other than mechanoreception was sought. Sound waves from a tuning fork (100 cps) and loud noises resulted in no detectable response by the intact, functioning sensillae. Addition of xylene to the tarsal and metatarsal sensillae caused no observable response. These sense organs functioned typically before and after application of the xylene. Pringle (1955) used the application of liquid xylene on slit sensillae as a criterion of possible chemoreceptive sensitivity.

DISCUSSION

The shape of phalangids is exaggerated as compared to that of most arthropods, and indeed other forms, in that the legs typically are extremely long and of small diameter compared to the small, oval body. Being without antennae the animal first encounters its environment predominately with its legs. For this reason, one might expect to find a variety of sensory organs here.

The legs are directed radially and upward from the animal in an arch so that the body is suspended in the middle third of the distance from the surface touched by the tarsi to the highest part of the arch of the leg. The body is therefore exposed only directly above and below. All joints of the legs operate essentially in one plane with respect to the body (dorso-ventral) except the trochanter-femur which, in combination with the coxa-trochanter, is capable of movement in a variety of dorso-ventral and anterio-posterior directions. It is at the femoro-trochanter junction that the leg may be autotomized. In nature, phalangids frequently are encountered with one or several legs missing. Legs grasped by predators or trapped during the molting process are readily shed in order to escape.

Sensillar supply is richest near this autotomy plane and diminishes to where the only type present on the distal half of the leg is the campaniform sensillium. These few isolated organs register stretch and compression pressure in the region of joints, *i.e.*, between leg segments or between the hinge-like series of tarsal articles. Presumably, stress in other than the plane of the joint movement would be detected mainly in the region of the autotomy plane, where groups of slit sensillae are oriented so as to respond to deformation from any direction (Figs. 1 and 2). The campaniform organs detect shock stress coming from ventral (about the tibiometatarsal joint) and dorsal or ventral directions (about the metatarsotarsal joint and adjoining tarsal articles). These organs apparently do not function to indicate, to the animal, position of the appendage while at rest or from contacts with the relatively stationary aspects of the environment (forest litter, tree trunks, etc.) since action potentials are picked up only when the normal limits of the joint are exceeded. Hence, they function as well-distributed alarm systems which "go off" when the safety of the limb and probably the animal are threatened.

SUMMARY

1. In the legs of phalangids thin areas in the exoskeleton occur in four basic arrangements: (a) solitary slit sensillae, (b) I- and L-shaped slits in clusters, (c) lyriform organs, and (d) circular or campaniform sensillae.

2. Evidence was obtained to indicate that at least the campaniform sensillae on the phalangid tarsus and metatarsus function as proprioceptors. Action poten-

tials, from grouped and isolated slit organs on the tibia and femur, were elicited but were less distinct as to electrical characteristics and function.

3. No response was seen upon application of sound waves and xylene to the campaniform sensillae.

LITERATURE CITED

Berlese, A., 1909. Gli Insetti, 1. Soc. Edit. Libraria, Milan.

Bertkau, P., 1878. Versuch einer naturlichen Anordnung der Spinnen nebst Bemerkungen zu einzelnen Gattungen. *Arch. Naturgesch.*, **44**: 354–510.

Gaubert, P., 1890. Note sur les organes lyriformes des Arachnides. *Bull. Soc. Philom. Paris, Ser. 8*, **2**: 47.

Gaubert, P., 1892. Recherches sur les organes des sens et sur le systeme tegumentaire, glandulaire et musculaire des appendices des Arachnides. *Ann. Sci. Nat.*, **13**: 31–185.

Hansen, H. J., 1893. Organs and characters in different orders of arachnids. *Entomol. Medd. udg af Ent. For. i Kjobenhavn'*, **4**: 137–251.

Hansen, H. J., and W. Sorensen, 1904. On Two Orders of Arachnida. Cambridge University Press.

Kaston, B. J., 1935. The slit sense organs of spiders. *J. Morph.*, **58**: 189–207.

Pringle, J. W. S., 1938. Proprioception in insects, I. A new type of mechanical receptor from the palps of the cockroach. *J. Exp. Biol.*, **15**: 101–113.

Pringle, J. W. S., 1955. The function of the lyriform organs of arachnids. *J. Exp. Biol.*, **32**: 270–278.

Salpeter, M. M., and Charles Walcott, 1960. An electron microscopical study of a vibration receptor in the spider. *Exp. Neurology*, **2**: 232–250.

Savory, T. H., 1962. Daddy Longlegs. *Sci. Amer.*, **207**: 119–128.

Snodgrass, R. E., 1935. Principles of Insect Morphology. McGraw-Hill Book Co., Inc., New York and London. 667 pp.

OBSERVATION ON THE ECOLOGY AND REPRODUCTION OF FREE-LIVING CONCHOCELIS OF PORPHYRA TENERA

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In a previous paper (Iwasaki, 1961) it was shown that free-living *Conchocelis* colonies produce normal monosporangia and monospores under short-day conditions (8–11 hours light daily); these monospores germinate into leafy thalli as they do in nature.

The free-living *Conchocelis* colonies cultured in continuous light produce only other *Conchocelis* colonies. It became then interesting to find how new *Conchocelis* colonies are produced. It was found that new colonies could be obtained from small pieces of *Conchocelis* filaments. However, the *Conchocelis* colonies grown in continuous light produce sporangia which are morphologically different from the normal sporangia produced under short-day conditions. Even though free spores were not found in continuous light, it was not excluded that new *Conchocelis* colonies could be produced by special spores produced under continuous light.

The present work was undertaken to study the type of sporangia formed by free-living *Conchocelis* colonies under long-day conditions, and the fate of their spores. It was found that spores are indeed formed and that they can originate new *Conchocelis* colonies, revealing, under these conditions, a new part of the life-cycle of *P. tenera*.

MATERIALS AND METHODS

The original material derives from mature leafy thalli collected at Matsukawa-ura Inlet located near Sendai in 1960. The *Conchocelis* colonies used for the present studies were obtained from carpospores produced by the leafy thallus passing through one life-cycle *in vitro*. This *Conchocelis* strain was a uni-algal culture, but accompanied by bacteria and yeast. Two treatments with ultraviolet light for 1–2 minutes, at intervals of one month, eliminated the bacteria but not the yeast. Two types of liquid media, the artificial medium ASP12NTA (Provasoli's medium), and the enriched sea water medium SWI, were employed for the experiments (Table I). The cultures were carried on mainly in screw-cap tubes (125 × 20 mm.) with 10 ml. of medium. When necessary, the hanging culture technique was employed.

RESULTS

I. *Types of sporangia produced in long-day conditions*

a) The "inflated spherical" cells

Small pieces of *Conchocelis* filament were inoculated in culture media and grown under different conditions. The first observation was made on the strain cultured at a window in subdued natural light (max. 500 ft.c.) from July to

Table I

Enriched sea water medium, SWI

Filtered sea water	1000 ml.
KNO_3	72.2 mg.
KH_2PO_4	8.8 mg.
Fe-EDTA (1:1 chelation)	0.5 mg. (as Fe).
"Tris Buffer"*	500 mg.
pH	7.8–8.0

* Tris (hydroxymethyl) amino methane (Sigma Company).

Artificial medium ASP12NTA

Distilled water	100 ml.	$Na_2SiO_3 \cdot 9H_2O$	15 mg.
NaCl	2.8 g.	B_{12}	0.02 μg.
$MgSO_4 \cdot 7H_2O$	0.7 g.	Biotin	0.1 μg.
$MgCl_2 \cdot 6H_2O$	0.4 g.	Thiamine	10 μg.
KCl	0.07 g.	P II metals*	1 ml.
Ca (as Cl)	40 mg.	S II metals**	1 ml.
$NaNO_3$	10 mg.	"Tris" buffer	0.1 g.
K_3PO_4	1 mg.	Nitrilotriacetic acid	10 mg.
Na_2 glycerophosphate	1 mg.	pH	7.8–8.0

* One ml. of P II metals contains: EDTA, 1 mg.; Fe (as Cl), 0.01 mg.; B (as H_3BO_3), 0.2 mg.; Mn (as Cl), 0.04 mg.; Zn (as Cl), 0.005 mg.; Co (as Cl), 0.001 mg.

** One ml. of S II metals contains: Br (as Na), 1.0 mg.; Sr (as Cl), 0.2 mg.; Rb (as Cl), 0.02 mg.; Li (as Cl), 0.02 mg.; I (as K), 0.001 mg.; Mo (as Na), 0.05 mg.

September at temperatures ranging from 20 to 28° C. Small plants of *Conchocelis* formed new lateral branches in a week. Some of the tips of branches swelled to form a more or less spherical cell about three weeks after inoculation. These cells became more deeply pigmented and gradually reached 11.4–14.0 μ in diameter (Plate I, A). Soon the spherical cells detached from the branches and became free spores, mostly spherical in shape (9.8–11.7 μ in diameter, Plate I, B). The spores begin to germinate after 3–7 days from the liberation. At first the spore forms a germ tube, then a delicate cross-wall appears between the original spore and the tube, and soon lateral branches form. These early filamentous germlings were usually quite tortuous. They did not orient to any particular direction in relation to light. Finally, the germlings grew into luxuriant branched filamentous thalli.

b) The special "sporangia"

In the culture grown at 17–19° C. and exposed to 16 hours fluorescent light (350 ft.c.), the *Conchocelis* colonies produced many sporangia (SI) as shown in Plate I, D–F. The sporangia are very similar to the sporangia produced under continuous light (Iwasaki, 1961, Fig. 4, p. 178). As mentioned in that paper, the sporangia produced under long-day conditions seemed to be different morphologically from the monosporangia produced under short-day conditions. The sporangia cells have thicker walls and the length of cells is usually about half their diameter.

c) The "strawberry-like" bodies

In the mass culture under the same conditions, very strange, round strawberry-like structures (70–80 μ in diameter) were found. These structures (Plate I, C)

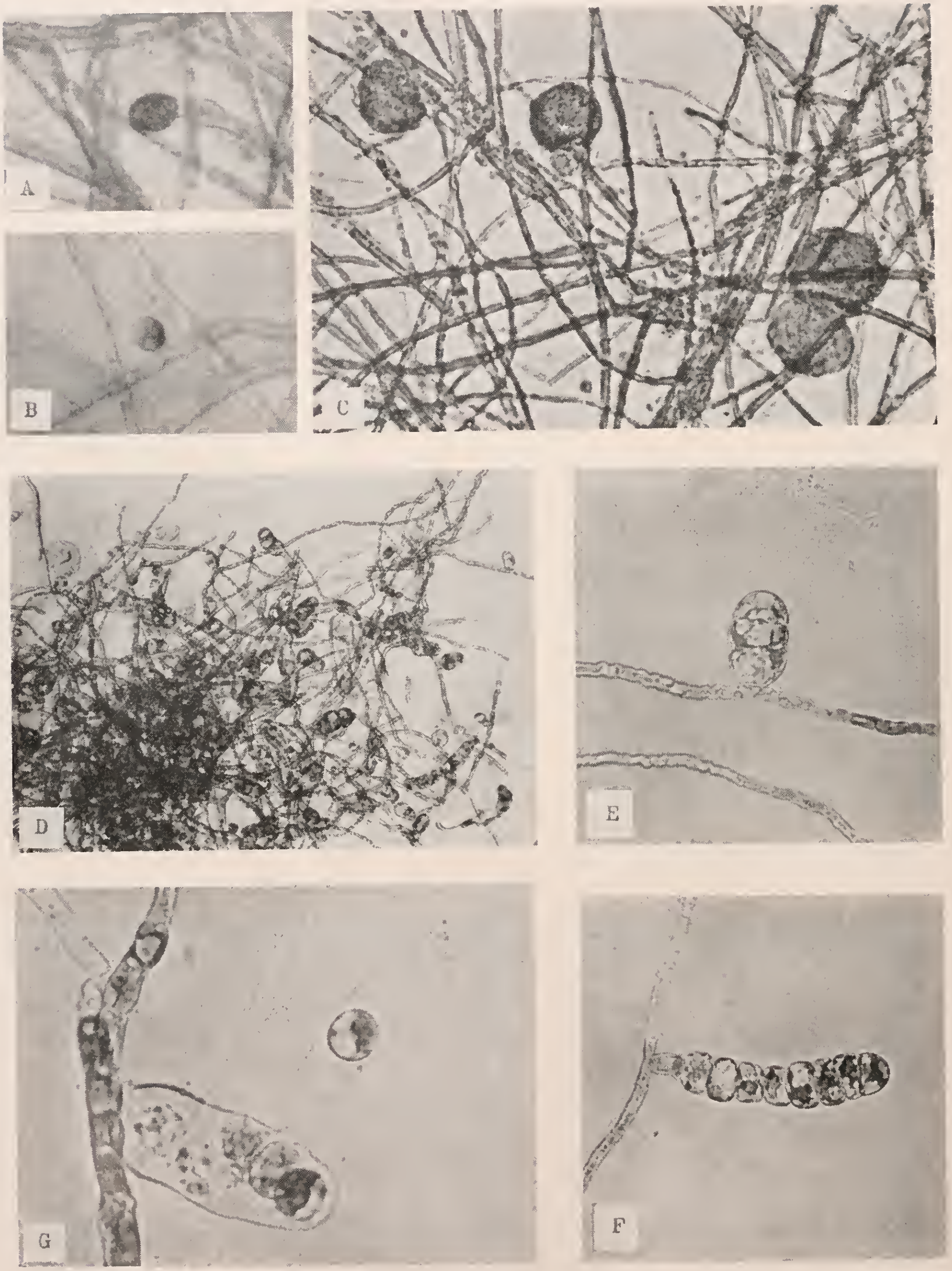

PLATE I

A, B. Inflated spherical cells (A) and liberated concho-monospore which germinate into *Conchocelis* thallus (B). × 256. C. Round strawberry-like structures formed on the *Conchocelis* branches. × 160. D, E, F, G. Sporangia (SI) formed in long-day conditions; D, × 96; E, × 192; F, × 160; G, liberation of the spores, × 384.

have numerous very slender colorless filaments, less than 1 μ broad, 10–20 μ in length, on the surface. At first they seemed to be some kind of parasitic fungi, but about a month after their appearance, many new filamentous *Conchocelis* colonies were found in the culture.

II. *Fate of the "spores" produced by the "strawberry-like" bodies*

Suspecting that these germlings might derive from the "strawberry-like" bodies, 16 of these structures were cut off with a scalpel from the branches, then cultured in hanging drops (ten structures) and in screw-cap tubes (six structures), respectively, under identical conditions (long day, 350 ft.c. fluorescent light). These structures, even after the separation from the branches, grew to the size of about 140 μ in diameter.

a-1) In two cultures in hanging drops, several germ tubes grew directly from the strawberry-like structure after 16 days. The germ tubes elongated, formed lateral branches, and then grew into *Conchocelis* colonies by repeated branching.

a-2) In two other cultures, after three weeks or more, the structures disintegrated, liberating several ameboid, unicellular spore-like bodies. Two of them germinated in a way similar to the carpospores produced by leafy thallus and grew into free-living *Conchocelis* colonies. Two kinds of *Conchocelis* filaments were observed, one thick (7.6–8.0 μ broad) and the other thin (4.0–5.0 μ broad). These two kinds of young *Conchocelis* were transferred to new medium, but they became infected by bacteria and the culture medium became cloudy. The thick-filamented *Conchocelis* grew nicely despite the bacterial infection and the culture medium became clear after three weeks. The thin-filamented *Conchocelis* bleached soon after transfer.

a-3) The other strawberry-like structures in hanging drops did not germinate and became covered by very slender dark brown filaments after a month or more.

b-1) The six structures inoculated in screw-cap tubes attached themselves immediately to the side wall of the tubes. After two weeks the internal cells of the structure produced several germ tubes which grew into normal *Conchocelis* (four cultures). One of these *Conchocelis* colonies formed monosporangia after 45 days, and soon liberated monospores that gave rise to leafy thalli reaching 2–3 mm. before they became pale and died under the same long-day conditions. Half of these monospores were isolated and also germinated into normal leafy thalli under short-day conditions (9 hours daily of incandescent light) at 13–15° C. Three other *Conchocelis* cultures formed very poor sporangia which did not liberate any spores, either under long-day or under short-day conditions (150–300 ft.c. fluorescent light for 270 days).

b-2) One of the strawberry-like structures disintegrated in two weeks but after a month, eight young *Conchocelis* colonies were found. Unfortunately, the observation of this experiment was done through the wall of the test tube, using a dissecting microscope, so the germination could not be followed in detail. It is probable that these young *Conchocelis* colonies originated from ameboid-shaped spores produced by the strawberry-like structure. These *Conchocelis* colonies formed many monosporangia and liberated a lot of monospores under short-day conditions. These monospores also germinated into normal leafy thalli.

III. *Fate of the "spores" of long-day sporangia (SI) under different light periods*

Half of the *Conchocelis* culture, which formed "parasporangia-type" sporangia (SI, see section Ib), were moved to short-day conditions (9 hours daily of 200 ft.c. incandescent light) at 13–15° C., and the other half were kept at 17–19° C. under fluorescent light of 200 ft.c. and a daily photoperiod of 14 hours. In about two weeks, the sporangia discharged many spores. The liberation of the spores was a little earlier in the short-day condition. The manner of spore liberation is shown in Plate I, G. These spores were taken up with a capillary pipette and inoculated in new culture media. The majority of spores did not germinate either under short-day or long-day conditions. In general a distinctly higher percentage of germination was observed in closely grouped spores than in less closely grouped clusters. Many abnormal pale germlings (Plate II, E, F), which soon bleached, were observed.

a) Under long-day conditions the spores gave rise to filaments but soon the original spore enlarged and divided, forming a sporangia-like body (Plate II, A). Sporangia-like bodies are also formed in the terminal part of filaments or as lateral growth on the singly-branched filaments (Plate II, B). After that the filaments became *Conchocelis* colonies and produced well developed luxuriant sporangia-like bodies (Plate II, B).

Some other spores continue to enlarge after liberation; occasionally they produce root-like projections and grow into globular bodies, about 110 μ in diameter (Plate II, C, D). Another group of spores enlarged, then grew into irregular-shaped "blades" as shown in Plate II, G.

b) The spores kept under short-day conditions produce short, colorless filamentous projections (about less than 100 μ). The projections may occasionally branch. The original spore enlarged while the filamentous projections elongated and became more deeply pigmented, then formed a cross-wall (Plate III, F). At this stage, the filamentous part bleached while the original spore continued to divide, forming a sporangia-like body composed of cells quite short and broad (Plate III, G). No more elongation and longitudinal division of germlings occurred even under these conditions which are suitable for the growth of leafy thallus. Some germlings produce robust branches near the original spore body, occasionally on the first sporangia-like branch, and grow into the aberrant plantlets as shown in Plate III, A–E. "Massive plantlets" (Plate III, I–K, L–O), irregular-shaped blades and abnormal germlings, as mentioned above, were also found in the culture under short-day condition.

DISCUSSION

New *Conchocelis* colonies can grow from small pieces of filaments (less than 1 mm. in length). Some of the new colonies, therefore, may derive from small pieces of filaments that are cut off naturally, especially in old cultures, and by shaking.

Inflated spherical cells are often formed at the tip of branches. These inflated cells were at first thought to be undeveloped monosporangia, but no leafy thalli developed from the spores found in these cultures. It is sure, therefore, that the spores derived from inflated cells are quite different functionally from the monospores that develop into leafy thallus. It is not clear yet whether or not the inflated cells are formed on definite branches. This asexual reproduction closely resembles

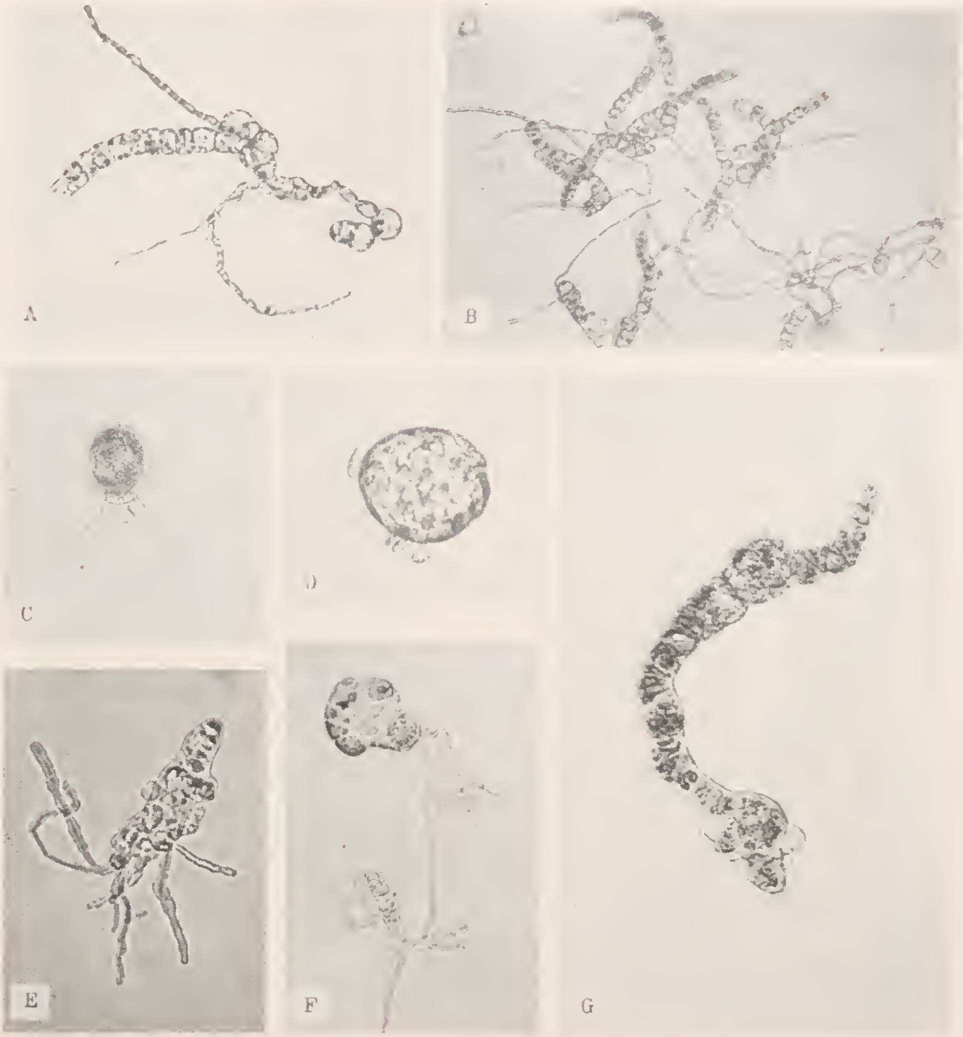

Plate II

Various germlings developed from "spores" originating from sporangia (S1) in 14 hours' illumination daily. A, B, *Conchocelis*-like plants; A, × 160, B, × 72. C, D, globular body, × 160. E, F, (Abnormal one?) × 160. G. Blade-like body, × 160.

that of *Rhodochorton.* It would seem fitting to call them "Concho-monospores" to specify that they originate new *Conchocelis* colonies and to differentiate them from the normal monospores which develop into leafy thalli.

It is not clear whether the strawberry-like structures are polysporangia or cystocarps. The difference observed in the germinative processes derived from isolated strawberry-like structures seems mainly due to the maturation stage of the structure. The strawberry-like structures matured normally on the *Conchocelis* branches, liberating two to eight spores which germinated into *Conchocelis* colonies.

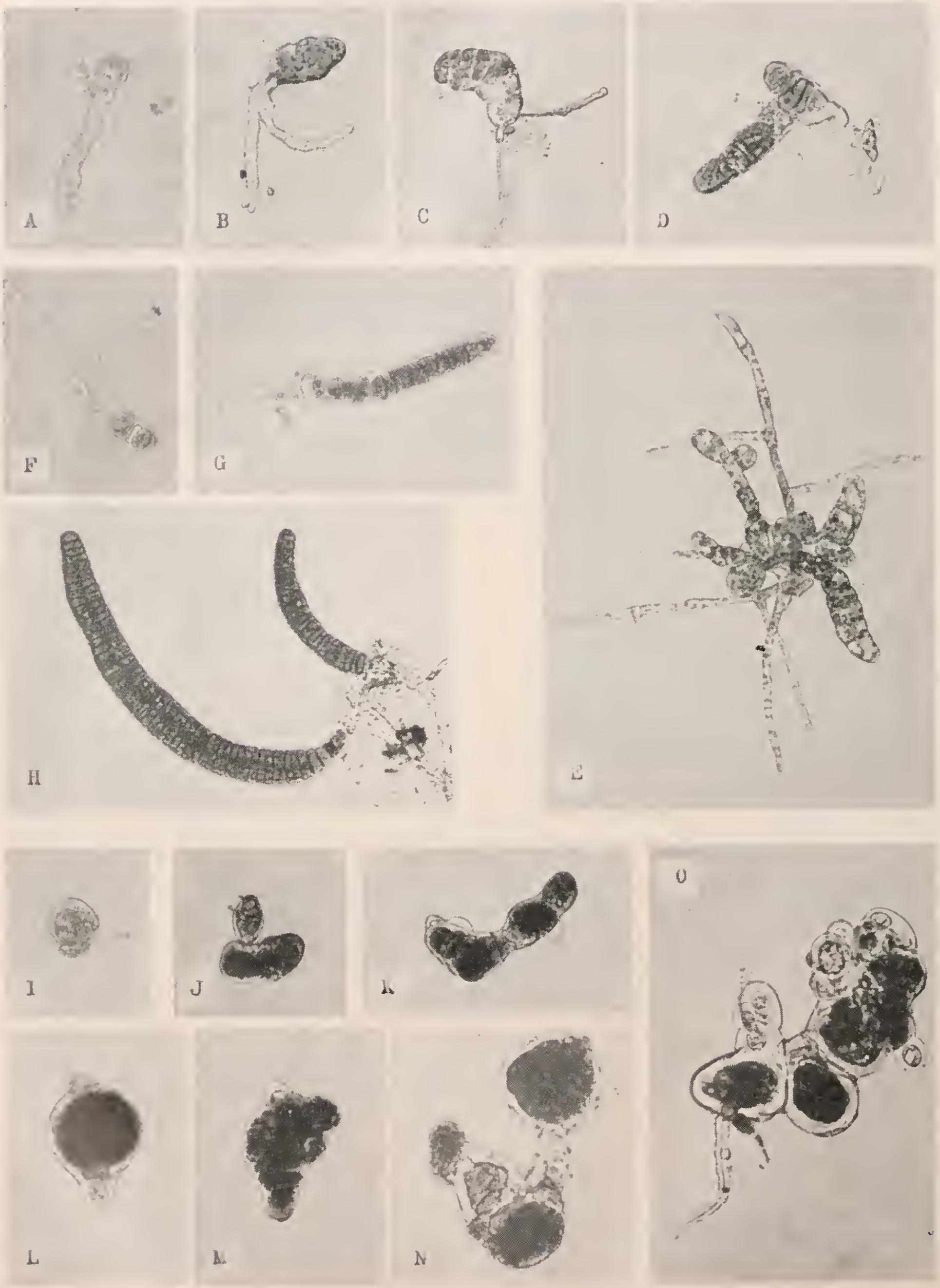

Plate III

Various germlings developed from "spores" originating from sporangia (SI) in 9 hours' illumination daily (except H). A–E, Plantlets developing from the "spores," × 160. E, × 192. F–G, Blade-like bodies, × 160. I–O, Massive plantlets, × 160. H, Young buds of leafy thalli developed from monospores, × 160.

These *Conchocelis* colonies formed monosporangia and liberated monospores which germinated into normal leafy thalli under short-day conditions.

One of the *Conchocelis* colonies derived from strawberry-like structures produced monosporangia and monospores which gave rise to leafy thalli (2–3 mm. in length) even in long-day conditions. This behavior of the *Conchocelis* phase in long-day conditions (16 hours daily, fluorescent light) had never been observed in our culture *in vitro*. Is this *Conchocelis* phase a special one or a mutant? More work is needed to solve these questions.

The "Concho-monospores" and strawberry-like structures have been observed at temperatures higher than 18° C. and under long-day conditions. It seems that the formation of inflated spherical cells and the strawberry-like structures is affected by photoperiodism and temperature.

The sporangia (SI) produced by *Conchocelis* colonies grown under long-day conditions resemble in function the plantlets that Kornmann used to start his cultures (1960). These sporangia produce spores which germinate into new *Conchocelis* colonies having well developed sporangia under long day, though their germination rate is considerably lower.

In the short-day condition, the spores develop into "plantlets" (ours) having a few or poor filaments (rhizoids). The "plantlets" are morphologically very similar to the sporangia that *Conchocelis* develops in long day but seem to be functionally different.

Although numerous "plantlets" with rhizoids appear in the culture of *P. pseudolinearis* (Plate IV), this type of "plantlet" without rhizoids has not been found in *P. tenera* cultures. The "plantlets" found in our cultures under short-day condi-

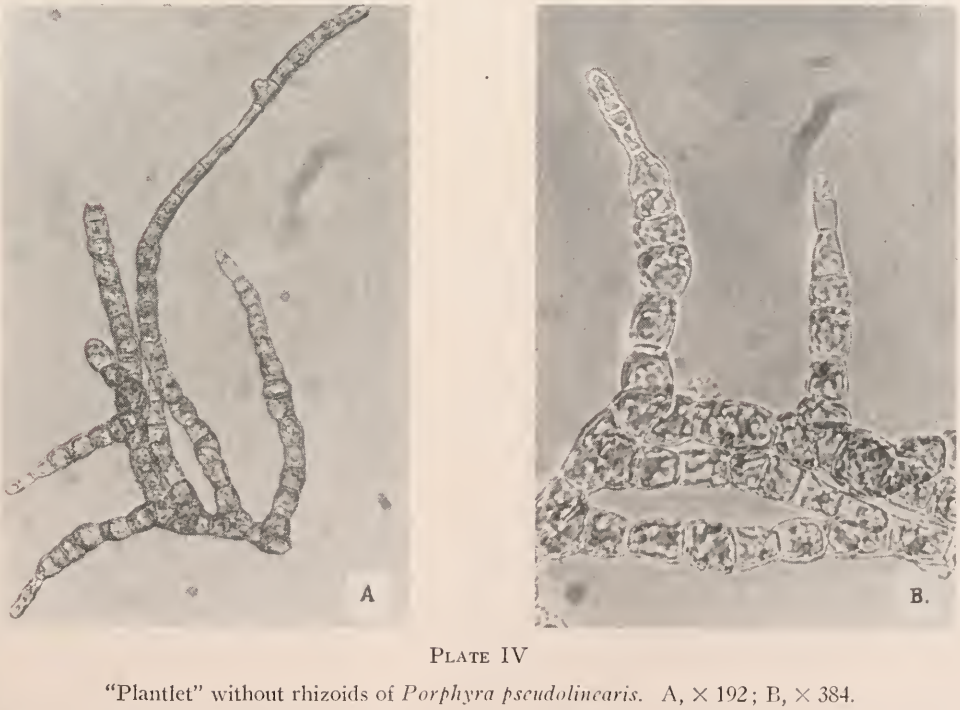

PLATE IV

"Plantlet" without rhizoids of *Porphyra pseudolinearis*. A, × 192; B, × 384.

tions are similar to the "plantlets" described by Drew (1954) in her culture of *P. umbilicalis.*

The blade-like bodies developed in short day (Plate III, F, G) look like young buds of leafy thalli but they do not elongate to more than 1 mm. in length and do not show longitudinal divisions (Plate III, F, G and H).

What are the globular and irregular blade-like bodies (Plate II, C, D and G) developed in long-day conditions, and what roles do they play in the life-cycle? And also, what are the blade-like bodies (Plate III, G) and "massive plantlets" (Plate III, I–O) in short-day conditions? Are they merely abnormal germinations of leafy thalli? The "plantlet" seen in Plate III, O, seems ready to liberate some unknown spores.

The variety of structures created under different light and temperature conditions shows that *P. tenera* has unusual power of adaptation to the environment, and more work is needed to solve some of the problems posed by the growth potencies of *P. tenera.*

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SUMMARY

1. Free-living *Conchocelis* colonies under long-day conditions produce several types of reproductive bodies from which new *Conchocelis* colonies originate, revealing a new part of the life-cycle.

2. Three reproductive structures were observed and described: (a) inflated spherical cells; (b) strawberry-like structures; (c) special "sporangia" (SI).

3. The inflated spherical cells discharge single spores which develop *Conchocelis* colonies.

4. The interior cells of the strawberry-like structures and unicellular spore-like bodies discharged by disintegration of the structure produce germ tubes, and grow into *Conchocelis* colonies.

5. These *Conchocelis* colonies produce monosporangia and liberate the monospores that germinate into normal leafy thalli under short-day conditions. The spherical cells and the strawberry-like structures were produced on *Conchocelis* branches at temperatures higher than 18° C. and under long-day conditions (subdued light).

6. The spores liberated from sporangia (SI) formed under long-day conditions develop into various plantlets: (a) *Conchocelis*-like plants, globular bodies and abnormal blades in long day (14 hours' illumination daily); (b) plantlets having short and root-like filaments, blade-like bodies and massive plantlets in short day (9 hours' illumination daily).

LITERATURE CITED

DREW, K. D., 1954. Studies in the Bangioidae III. The life-history of *Porphyra umbilicalis* (L.) Kütz. var. *laciniata* (Lightf.) J. Ag. *Ann. Bot., N. S.*, **18**: 183–211.

KORNMANN, P., 1960. Von *Conchocelis* zu *Porphyra. Helgolander Wiss. Meeresunters.,* **7**: 189–193.

IWASAKI, H., 1961. The life-cycle of *Porphyra tenera in vitro. Biol. Bull.,* **121**: 173–187.

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ENVIRONMENTAL AND TISSUE TEMPERATURES OF SOME TROPICAL INTERTIDAL MARINE ANIMALS

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Intertidal marine animals may be subjected over short periods of time to a broad spectrum of physical conditions. High temperature and desiccation are likely to be important factors for tropical intertidal forms. The importance of these factors has been noted in determining vertical distribution of gastropods, by Broekhuysen (1940). Although they are poikilotherms, it cannot be assumed that the body temperatures of intertidal forms are the same as the meteorological values of the micro-climate which they inhabit. The effects of insolation and evaporation on tissue temperatures of insects and other terrestial arthropods are well known (Gunn, 1942; Parry, 1951; Wigglesworth, 1948; Edney, 1951, and many others). The body temperatures of intertidal marine animals have, however, received little attention, except for a study by Southward (1958) on northern intertidal forms. Southward found body temperatures in many cases to be higher than the local meteorological values, due to retention of sea water and warming by sunlight. In tropical forms one would expect the effect of insolation to be pronounced. The preference of certain intertidal animals for shaded areas has been noted by Lewis (1960) and Broekhuysen (1940).

METHODS

Temperatures were measured with fine wire, copper/constantine, thermocouples insulated with lacquer. The e.m.f. was measured against a reference bath of ice and water in an insulated Thermos flask at 0° C. The recording instrument was a portable potentiometer manufactured by Pye Instruments Ltd., Cambridge, England. An accuracy of ±.25° F. is claimed by the manufacturer. Wet and dry bulb air temperatures were measured with a sling psychrometer.

Three species were selected for study: the barnacle, *Tetraclita squamosa,* the limpet, *Fissurella barbadensis* and the gastropod, *Nerita tesselata.* These species were abundant at the site chosen, a flat platform of beach rock which is fully exposed at low tide. Body temperatures were measured by thrusting the thermocouple tip under the operculum of *Nerita,* through the apical hole of *Fissurella* and between the plates of the carapace of *Tetraclita.* Temperatures of a black body and an inanimate body were also measured. The black body was a piece of soft black mastic and the inanimate body, the shell of a *Nerita,* stuffed with mastic. The deflection of the galvonometer was noted immediately, and each animal was used only for a single temperature measurement.

RESULTS

(a) *Climate of the intertidal zone*

The observed temperatures of the intertidal zone, recorded in Tables I–VIII, clearly indicate that air temperatures measured on the location do not give a complete assessment of the microclimate. The difference between air temperature in a shaded crevice and that of a black body on the rock surface may, for example, be as great as 23° F. on a hot sunny day. On a day with no sun the range of temperatures is not as great. The difference between air shade temperature and black body temperature was only 4° F. on an overcast day.

Throughout the course of observations the temperatures taken in various places measured formed an ascending sequence from wet bulb temperature to black body temperature. The lowest temperatures recorded were those of the wet bulb thermometer, which was cooled by rapid evaporation. The next lowest was the air shade temperature, measured in sheltered crevices close to the water or to a wet rock surface. Dry bulb temperatures were slightly higher than air shade temperatures but lower than air sun temperatures. The differences between these three air temperatures were due to the effects of evaporation and heating

TABLE I

Observations of temperatures in the intertidal zone and of tissue temperatures of barnacles, April 24, 1962, 1200–1300 hours

Air temp. Shade	Air temp. Sun	Dry bulb	Wet bulb	Sea temp.	Rock surf.	Inanim. body	Black body	Tissue temp.
82.2	88.0	87.2	73.0	86.4	95.1	101.0	105.2	94.8
85.3	90.0	86.5	73.5	87.0	95.5	98.8	107.8	101.0
84.3	90.5	86.3	72.3	87.4	95.3	98.8	105.6	99.6
86.4	92.2	86.0	72.5	88.6	95.1	99.6	108.8	97.4
85.3	89.0	86.0	72.7	88.2	95.2	99.2	108.2	99.2
84.3	90.8	85.0	73.5	89.0	94.3	102.8	112.3	102.4
Mean 84.6	90.1	86.2	72.9	87.8	95.1	100.0	107.9	99.1

Weather Remarks: No clouds, bright sun, fresh breeze, low tide 1200 hours.

TABLE II

Observations of temperatures in the intertidal zone and of tissue temperatures of barnacles, May 8, 1962, 1300–1400 hours

Air temp. Shade	Air temp. Sun	Dry bulb	Wet bulb	Sea temp.	Rock surf.	Inanim. body	Black body	Tissue temp.
87.0	87.8	88.0	76.2	94.8	97.4	105.8	110.0	96.8
85.8	88.4	87.5	76.5	95.3	97.2	101.5	110.0	100.8
89.2	91.3	87.5	76.0	94.8	97.2	102.4	111.6	102.0
86.4	90.8	87.0	76.2	96.1	99.2	103.2	109.6	101.0
85.0	91.3	87.0	76.5	95.5	96.4	98.0	106.0	96.8
Mean 86.7	89.9	87.4	76.3	95.3	97.5	102.2	109.4	99.5

Weather Remarks: 5/10 cloud cover, moderate breeze, low tide 1240 hours.

TABLE III

Observations of temperatures in the intertidal zone and of tissue temperatures of barnacles, July 4, 1962, 1150–1250 hours

Air temp. Shade	Air temp. Sun	Dry bulb	Wet bulb	Sea temp.	Rock surf.	Inanim. body	Black body	Tissue temp.
79.4	79.4	77.3	77.3	83.4	82.0	82.0	83.2	83.8
79.8	79.6	82.6	80.0	83.2	80.5	82.4	86.0	86.4
81.3	80.8	83.3	81.0	83.3	81.8	82.8	87.0	83.8
82.0	82.0	81.2	78.5	83.2	82.0	84.3	87.2	84.0
81.5	82.4	82.0	78.8	83.4	81.5	84.0	87.6	85.0
81.8	82.2	82.0	79.0	83.4	82.0	85.0	87.2	84.3
Mean 81.2	81.4	81.6	79.1	83.5	81.7	83.7	86.6	84.5

Weather Remarks: 10/10 cloud cover after light rain, very light wind, low tide 1000 hours.

TABLE IV

Observations of temperatures in the intertidal zone and of tissue temperatures of gastropods, May 21, 1962, 1100–1200 hours

Air temp. Shade	Air temp. Sun	Dry bulb	Wet bulb	Sea temp.	Rock surf.	Inanim. body	Black body	Tissue temp.
88.4	93.6	86.5	76.5	93.0	95.2	104.6	111.2	99.0
88.8	89.6	86.0	76.0	93.0	95.1	103.6	111.4	96.1
88.6	88.4	89.0	77.5	93.0	99.8	101.3	105.4	92.4
88.6	93.0	88.0	77.0	93.0	99.6	105.4	112.0	95.5
92.2	91.0	90.1	78.5	93.0	99.2	105.6	114.0	95.5
90.5	91.0	88.5	77.5	93.0	104.4	106.0	116.8	94.0
Mean 89.5	91.1	88.0	77.2	93.0	98.9	104.4	110.1	95.7

Weather Remarks: 2/10 cloud cover, fresh breeze, low tide 1102 hours.

TABLE V

Observations of temperatures in the intertidal zone and of tissue temperatures of gastropods, July 4, 1962, 1150–1250 hours

Air temp. Shade	Air temp. Sun	Dry bulb	Wet bulb	Sea temp.	Rock surf.	Inanim. body	Black body	Tissue temp.
79.4	79.4	77.3	77.3	83.4	82.0	82.0	83.2	82.2
79.8	79.6	82.6	80.0	83.2	80.5	82.4	86.0	82.2
81.3	80.8	83.3	81.0	83.3	81.8	82.8	87.0	82.4
82.0	82.0	81.2	78.5	83.2	82.0	84.3	87.2	83.6
81.5	82.4	82.0	78.8	83.4	81.5	84.0	87.6	83.6
81.8	82.2	82.0	79.0	83.4	82.0	85.0	87.2	82.0
Mean 81.2	81.4	81.6	79.1	83.5	81.7	83.7	86.6	82.6

Weather Remarks: 10/10 cloud cover after light rain, very light wind, low tide 1000 hours.

TABLE VI

Observations of temperatures in the intertidal zone and of tissue temperatures of gastropods, May 8, 1962, 1300–1400 hours

Air temp. Shade	Air temp. Sun	Dry bulb	Wet bulb	Sea temp.	Rock surf.	Inanim. body	Black body	Tissue temp.
87.0	87.0	88.0	76.2	94.8	97.4	105.8	110.0	85.0
85.8	88.4	87.5	76.5	95.3	97.2	101.5	110.0	86.0
89.2	91.3	87.5	76.0	94.8	97.2	102.4	111.6	85.0
86.4	90.8	87.0	76.2	96.1	99.2	103.2	109.6	82.4
85.0	91.3	87.0	76.5	95.5	96.4	98.0	106.0	82.8
Mean 86.7	89.9	87.4	76.3	95.3	97.5	102.2	109.4	84.2

Weather Remarks: 5/10 cloud cover, moderate breeze, low tide 1240 hours.

TABLE VII

Observations of temperatures in the intertidal zone and of tissue temperatures of limpets, April 10, 1962, 1300–1400 hours

Air temp. Shade	Air temp. Sun	Dry bulb	Wet bulb	Sea temp.	Rock surf.	Inanim. body	Black body	Tissue temp.
83.2	86.0	82.3	72.5	80.0	85.0		96.7	88.8
81.0	84.5	82.3	72.5	80.0	86.0		98.8	86.8
80.5	86.8	82.3	72.5	80.0	85.5		97.5	87.2
Mean 81.6	85.8	82.3	72.5	80.0	85.5		97.6	87.6

Weather Remarks: 8/10 cloud cover (measurements made during sunny periods), strong breeze, low tide 1346 hours.

TABLE VIII

Observations of temperatures in the intertidal zone and of tissue temperatures of limpets, July 4, 1962, 1150–1250 hours

Air temp. Shade	Air temp. Sun	Dry bulb	Wet bulb	Sea temp.	Rock surf.	Inanim. body	Black body	Tissue temp.
79.4	79.4	77.3	77.3	83.4	82.0	82.0	83.2	85.5
79.8	79.6	82.6	80.0	83.2	80.5	82.4	86.0	85.5
81.3	80.8	83.3	81.0	83.3	81.8	82.8	87.0	86.0
82.0	82.0	81.2	78.5	83.2	82.0	84.3	87.2	85.8
81.5	82.4	82.0	78.8	83.4	81.5	84.0	87.6	86.4
81.8	82.2	82.0	79.0	83.4	82.0	85.0	87.2	86.0
Mean 81.2	81.4	81.6	79.1	83.5	81.7	83.7	86.6	85.9

Weather Remarks: 10/10 cloud cover after light rain, very light wind, low tide 1000 hours.

by the sun. Air in shaded crevices received no sunlight and was cooled by evaporation from the water surface. Dry bulb temperatures were taken in the shade, while the air sun temperatures, measured with thermocouples, were not subject to the above cooling influences.

Sea temperatures varied considerably and were observed to be above or below air sun temperature, depending upon the height of the tide. This was due to the fact that measurements were made in tide-pools which, at very low tides, were heated up by the sun and at higher water levels received periodic flushing from the sea. The temperatures of the rock surface were always several degrees above air temperatures, but varied with the degree of wetness.

The two extraneous elements introduced, the inanimate body and the black body, both produced temperatures higher than those measured elsewhere. The black body absorbed the maximum amount of radiation and had invariably the highest observed temperature. The observed temperatures of the inanimate body were lower than the black body but consistently higher than air temperatures. The source of heating above air temperature was presumably mainly the incoming radiation.

(b) *Tissue temperatures*

The results of observations of temperatures relating to the barnacles are shown in Tables I, II and III. Under conditions of full sunlight and a fresh breeze the body temperatures of the barnacles rose 9° F. above that of the air sun temperature. Body temperatures were also 4° F. higher than rock surface temperatures, but below those of the inanimate body and black body of 0.9° and 8.8° F., respectively.

On a partly cloudy day with moderate breeze, tissue temperatures of barnacles were above air sun temperatures by 9.4° and above rock surface temperatures by 2° F. Tissue temperatures were 2.7° lower than inanimate body and 9.9° F. lower than the temperatures of the black body.

Table III contains the observations on a completely overcast day. Temperatures were recorded shortly after a light rain. On this day tissue temperatures were only 3.1° above air sun temperatures and 2.8° F. above the rock surface. The temperatures of the inanimate body were 0.8° lower than tissue temperatures. Black body temperatures were only 2.1° F. higher than tissue temperatures.

On all three days body temperatures of barnacles were above air temperature. The differences were greatest on sunny days and considerably less on an overcast day. It is apparent, then, that the animals absorbed heat from incoming radiation. Body temperatures on sunny days rose nearly as high as the temperature of an inanimate body of the same size and color but not as high as a black body which absorbs the maximum amount of radiation.

The results of observations relating to the gastropod are shown in Tables IV, V and VI. On a day of full sunlight with a fresh breeze, tissue temperatures were 4.6° above the air sun temperature but below those of the rock surface, inanimate body and black body by 3.2°, 8.7° and 14.4° F., respectively.

On a partly cloudy day, body temperatures of the gastropods in shaded crevices were below shade air temperatures by 2.5° and sun air temperatures by 5.7° F. The body temperatures were far below that of an inanimate body by 18° F.

On a cloudy overcast day the difference between tissue temperature and environmental temperature was much less marked. Air sun temperature was 0.6° lower than tissue temperature, and inanimate body and black body temperatures were only 1.1° and 4° F. higher than tissue temperatures.

Thus, on the two sunny days the tissue temperatures of *Nerita* were very close to or below those of the ambient air temperatures. On neither day did they rise as high as the rock surface or inanimate body temperatures. While those specimens in sheltered crevices absorbed little of the incoming radiation, it is apparent that those exposed to the sun do not heat up in spite of radiation absorption.

The results of observations relating to limpets are shown in Tables VII and VIII. On a partly cloudy day, temperatures were recorded during sunny periods. Tissue temperatures were 1.8° above air sun temperatures and 2.1° F. above rock surface temperatures. Black body temperatures were 10° above tissue temperatures.

On an overcast day tissue temperatures were above air sun temperatures, rock surface and inanimate body temperatures by 4.5°, 4.2° and 2.2° F., respectively, but below that of the black body by 0.7° F.

On both days, tissue temperatures were above air temperatures and rock surface temperatures, but below those of the black body. It is apparent that this species is absorbing incoming radiation, but on a sunny day the body heat rises only slightly above air temperature.

Discussion

It is evident from the above results that tissue temperatures of animals living in the intertidal zone are not the same as the ambient air temperatures during exposure. Southward (1958) has attributed the rise in body temperature above sea and air temperatures of intertidal animals at Plymouth to warming by sunlight. Clearly this effect will be much more marked in the tropical forms.

Our results indicate, however, that intertidal animals do not absorb radiation as do inanimate bodies or black bodies. Thus, on a sunny day the tissue temperatures of barnacles are below black body temperatures by 8.8°, the gastropod *Nerita* by 14.1° and limpets by 10° F. Similarly, they were all below the temperature of an inanimate body.

It is thus apparent that there is some factor acting to reduce the heating effect of the sun. The most obvious cooling mechanism is that of evaporation. The effects of evaporation from the cuticle of insects have already been noted. In some insects evaporation may cool the body temperature several degrees C. below the air temperature (Gunn, 1942). It thus seems likely that a similar mechanism may be operative on intertidal animals.

In barnacles the plates of the carapace are tightly closed during exposure. There thus seems little opportunity for evaporation to occur in this species after the initial drying of the external shell. In *Nerita*, however, the foot attached to the substrate remains moist during exposure and the aperture of the shell is never pressed tightly downwards. When a specimen is lifted off the rock, the operculum closes and a drop of water remains in the aperture. This drop feels distinctly cool when placed on the skin. The temperature of the drop measured by a thermocouple did in fact show it to be identical with body temperature of the specimen. In limpets the shell is not firmly pressed against the substrate, so that evaporation can take place around the mantle. Furthermore, in *Fissurella* there is an apical hole through which evaporation can also take place.

In barnacles there is apparently the least opportunity for evaporation to take place, and observed tissue temperatures of this species were closer to those of the

TABLE IX

Water loss in Nerita during exposure on a cloudy day

Spec. no.	Wt. before exposure	Wt. after 1-hr. exposure	Wt. after 2-hr. exposure	Wt. after 4-hr. exposure	Wt. after 6-hr. exposure	Water loss 6 hr.
1.	24.056	24.051	24.046	24.035	24.026	0.030
2.	23.947	23.938	23.930	23.915	23.906	0.041
3.	25.713	25.709	25.703	25.692	25.683	0.030
4.	21.568	21.561	21.557	21.551	21.545	0.023
5.	23.774	23.763	closed			
6.	22.429	22.416		22.398	22.391	0.038
7.	21.507	21.504	21.501	21.493	closed	

inanimate body and black body, than the tissue temperatures of the limpets and gastropods.

Low body temperatures were most striking in the gastropod *Nerita*. In this species the tissue temperatures on a sunny day were lower in relation to environmental temperatures than in the other two species. If evaporative cooling is more effective in this species, then there will be a considerable loss of water during exposure, and hence a loss in body weight. Gowanloch (1926) has demonstrated heavy water loss of northern gastropods during exposure.

The losses in weight of animals exposed in full sunlight and on a cloudy day are shown in Tables IX and X. Each animal was placed on a glass dish with the operculum open and the foot attached to the glass. The glass with animal was weighed to the nearest 0.001 gram before exposure, and again at hourly intervals. Only those animals which kept their opercula open during the exposure period were considered.

The results of Tables IX and X indicate loss of water both in sunlight and on a cloudy day. There is a marked difference in the rate of evaporation, however. On a cloudy day there was a loss of less than 0.05 gm. of water in six hours. On a sunny day the loss in only two hours was between 0.05 and 0.1 gm. It would appear, then, that this species has an effective control over its body temperature by losing water during the exposure period in the intertidal zone through evaporation.

TABLE X

Water loss in Nerita during exposure on a sunny day

Spec. no.	Wt. before exposure	Wt. after 1-hr. exposure	Wt. after 2-hr. exposure	Water loss, 1 hr.	Water loss, 2 hr.
1.	29.161	29.101	closed	0.050	
2.	22.916	22.882	22.863	0.034	0.053
3.	24.167	24.120	24.100	0.047	0.067
4.	23.607	23.540	closed	0.067	
5.	21.554	21.498	21.471	0.056	0.083
6.	20.727	20.703	closed	0.024	

The difference among the three species studied, in resisting increase in body temperatures during exposure, suggests a relation to the vertical distribution of the three forms. At the site chosen, *Fissurella* and *Tetraclita* live at approximately the mid-tide level, while *Nerita,* which has the greatest control over its body temperature, is found at high tide (Lewis, 1960). Thus, the ability of intertidal forms to regulate their body temperatures may, in the tropics, have a bearing on intertidal zonation.

SUMMARY

1. Body temperatures of three common intertidal animals were measured with fine wire thermocouples.

2. Body temperatures were observed to be considerably above ambient air temperatures on a hot sunny day.

3. The difference between body temperatures and ambient air temperatures was less marked on a cloudy day.

4. Evaporative cooling was apparently the mechanism for lowering body temperatures. This process was most effective in the gastropod, *Nerita.*

5. A relationship between ability to regulate body temperature by evaporation and intertidal zonation is suggested.

LITERATURE CITED

Broekhuysen, C. J., 1940. A preliminary investigation of the importance of desiccation, temperature and salinity as factors controlling the vertical distribution of certain marine gastropods in False Bay, South Africa. *Trans. Roy. Soc. S. Africa,* **28**: 255–292.

Edney, E. B., 1951. The evaporation of water from woodlice and the millipede *Glomeris. J. Exp. Biol.,* **28**: 91–115.

Gowanloch, J. N., 1926. Contributions to the study of marine gastropods. II. The intertidal life of *Buccinum undatum,* a study in non-adaption. *Contr. Canad. Biol., N.S.,* **3**: 169–177.

Gunn, D. L., 1942. Body temperatures in poikilothermal animals. *Biol. Revs.,* **17**: 293–314.

Lewis, J. B., 1960. The fauna of rocky shores of Barbados, West Indies. *Canad. J. Zool.,* **38**: 391–435.

Parry, D. A., 1951. Factors determining the temperatures of terrestial arthropods in sunlight. *J. Exp. Biol.,* **28**: 445–462.

Southward, A. J., 1958. Note on temperature tolerances of some intertidal animals in relation to environmental temperatures and geographical distribution. *J. Mar. Biol. Assoc.,* **37**: 49–66.

Wigglesworth, V. B., 1948. The insect cuticle. *Biol. Revs.,* **23**: 408–451.

ACID AND ALKALINE PHOSPHATASE CHANGES ASSOCIATED WITH FEEDING, STARVATION AND REGENERATION IN PLANARIANS[1]

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The acid phosphatase activity in planarian gastrodermal cells has been reported to increase almost immediately following the ingestion of food (Rosenbaum and Rolon, 1960; Jennings, 1962b). Acid phosphatase has also been implicated in the early stages of digestion in the food vacuoles of paramecia (Müller and Törö, 1962) and of amebae (Müller, Toth and Törö, 1962). These results suggest an analogy between the food vacuoles of lower animals and the lysosomes (de Duve, 1961) of higher animals. Lysosomal enzymes are thought to be involved not only in the digestion of exogenous materials but also in the autolytic degradation of tissues in such processes as developmental involution and metamorphosis. It seems likely, therefore, that acid hydrolases may play a similar role in the gradual disappearance of digestive and reproductive organs during prolonged starvation in planarians, as well as in the provision of raw materials for the early stages of regeneration following transection, before the ingestion of exogenous food stuffs can be resumed.

Less is known about the significance of alkaline phosphatase than of acid phosphatase, although its frequent localization in absorptive epithelia in higher forms is suggestive of a role in the phosphorylation of certain compounds prior to their transport across membranes. High levels of alkaline phosphatase activity have been observed in several organs of planarians, including protonephridia (Danielli and Pantin, 1950), resting neoblasts (Pedersen, 1959) and nervous and muscular tissues (Gazso, Török and Rappay, 1961). Jennings (1962b) has recently reported the appearance of alkaline phosphatase in planarian gastrodermal cells several days following food ingestion, and he suggests that it may be concerned with the release of energy needed for secretion of the various digestive enzymes and for the absorption of the products of digestion from the food vacuoles.

The object of the present work was to extend the above-mentioned observations to later stages of digestion as they are succeeded by starvation effects, and to the tissue reconstruction involved in regeneration following transection.

MATERIALS AND METHODS

All observations were made on specimens of *Dugesia tigrina* obtained from the Carolina Biological Supply Company. Frozen sections were used instead of paraf-

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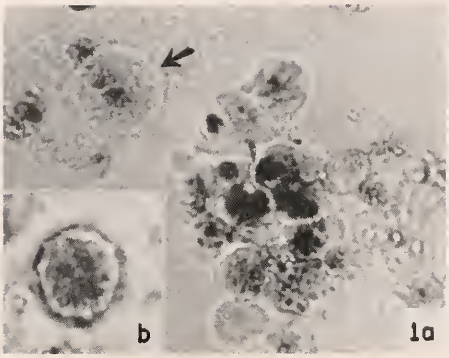
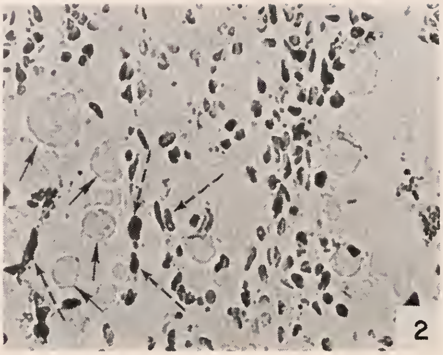
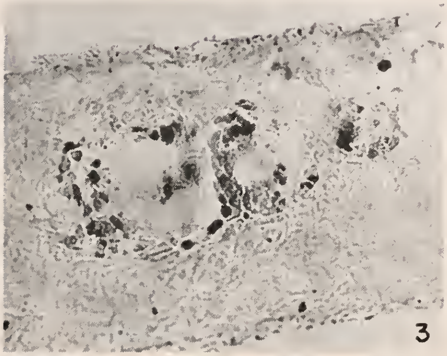
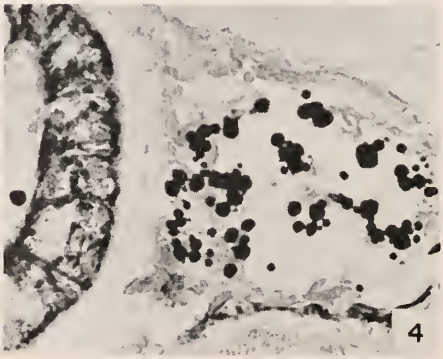
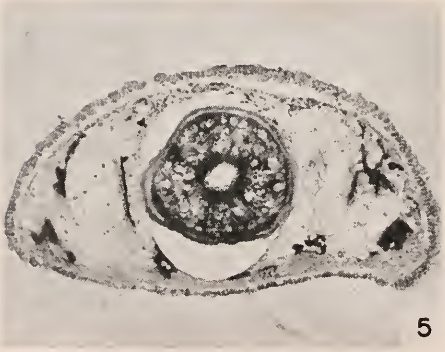
[1] Supported by grants from the National Institutes of Health (C-3996 and A-1699) and the National Science Foundation (NSF-GB-223 and NSF-GA-282).











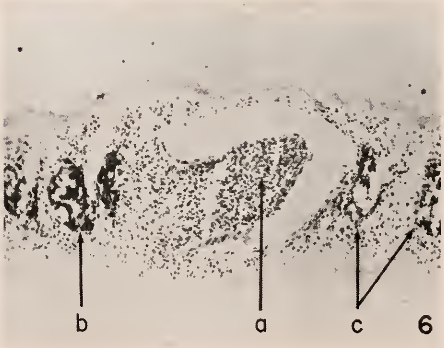


FIGURE 1a. Acid phosphatase activity in gastrodermal cells 24 hours after ingestion of cooked egg yolk. Arrow points to a gastrodermal cell containing two food vacuoles. 900 ×.

FIGURE 1b. Target form of gastrodermal cell, with acid phosphatase activity in cell membrane and in central core, two days after ingestion of cooked egg yolk. 1350 ×.

FIGURE 2. Eight days after ingestion of cooked egg yolk. The gastrodermal cells (solid arrows) are free of acid phosphatase activity and the gut region is being invaded by neoblasts (dotted arrows) with high acid phosphatase activity. 675 ×.

FIGURE 3. Thirty days after ingestion of cooked egg yolk. The intestinal epithelium is densely infiltrated by neoblasts with moderately high acid phosphatase activity. 300 ×.

FIGURE 4. Alkaline phosphatase activity in food vacuoles 24 hours after ingestion of cooked egg yolk. 300 ×.

fin-embedded sections, despite the better cytological preservation of the latter, because of the desire to preserve enzyme activity. This is especially important in the case of acid phosphatase, which undergoes considerable inactivation even when low-melting-point paraffin is used.

After a ten-day period of starvation (to eliminate the enzyme changes associated with previous feeding) the worms were allowed to feed on cooked egg yolk until satiated, and were then removed to fresh medium containing no food. At varying intervals after feeding, worms were slowly chilled to 4° C. and then fixed for 12–24 hours at 4° C. in 10% neutral formalin containing 1% calcium chloride. The fixed specimens were rinsed for 1–2 hours in distilled water at 4° C., then blotted and embedded in 10% gelatin, in the following manner. Melted gelatin was layered in the bottom of a small beaker and chilled until firm. Specimens were placed on top of the solidified gelatin, covered with a layer of melted gelatin and the beaker placed in a freezer until the gelatin had solidified. The gelatin was then cut into blocks, each containing a single worm; the blocks were mounted on cryostat object holders and frozen with dry ice or Freon. Sections were cut at 8 microns in a Pearse cryostat, mounted on chilled slides and air-dried. Acid and alkaline phosphatase activities were visualized by the methods of Gomori (1952).

For the studies on phosphatase changes during regeneration, planarians were starved for 10 days, the pharynx was carefully removed with dissecting needles, and the worm was cut transversely into equal halves which were allowed to regenerate in food-free medium. Each half regenerated its missing structures, including a pharynx, and the results to be described were identical in organisms regenerating from anterior and posterior halves.

RESULTS

Twenty-four hours after the ingestion of cooked egg yolk there was a moderately high level of acid phosphatase activity in the gastrodermal cells (Fig. 1a). This confirms the reports of Rosenbaum and Rolon (1960) and of Jennings (1962b). The enzyme reaction product at this time was in the form of small granules which gradually increased in size and numbers until they filled the entire cell by the second day after ingestion of food. At this time some of the gastrodermal cells had a "target" appearance, with intense acid phosphatase activity in the cell membrane, separated by a clear zone from the central core of enzyme activity (Fig. 1b).

Acid phosphatase activity in the gastrodermal cells diminished rapidly after the second day following food ingestion; by the fourth day many of the cells had no demonstrable activity, though minimal activity persisted in a few cells up to seven days. Coincident with the decline in acid phosphatase activity in the gastrodermal cells there was a striking increase in the activity of this enzyme in the neoblasts. By the eighth day there was a definite invasion of the gut region by acid phosphatase-rich neoblasts (Fig. 2). Thirty days after the ingestion of food the walls of

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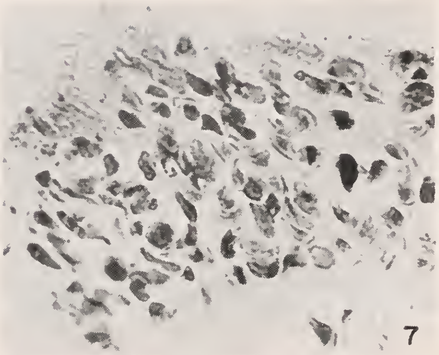
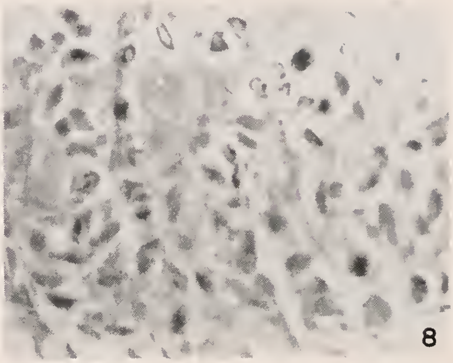
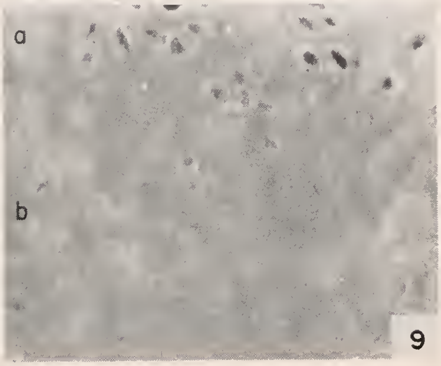
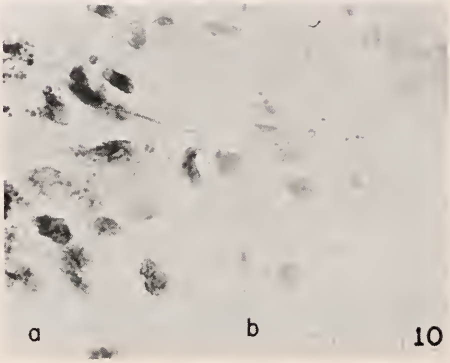
FIGURE 5. Alkaline phosphatase activity in pharynx, nerve fibers and glands after 60 days' starvation. 115 ×.

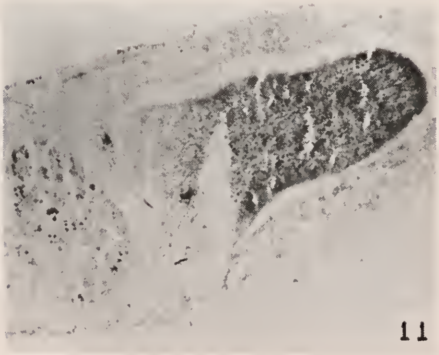
FIGURE 6. Regenerating planarian 6 days after transection (longitudinal section). The regenerating pharynx (a) is densely infiltrated with neoblasts having acid phosphatase activity, as are the original gut (b) and the regenerating gut (c). 75 ×.











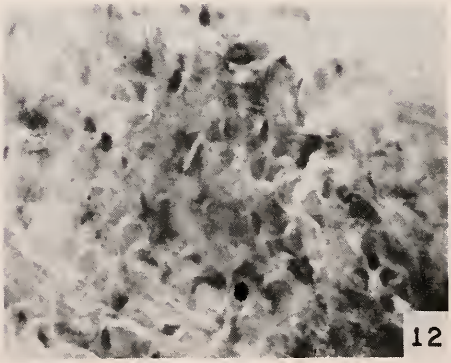


FIGURE 7. Higher magnification of cells of regenerating pharynx in Figure 6, showing neoblasts with acid phosphatase activity. 675 ×.

FIGURE 8. Phase contrast photograph of neoblasts in "inactive" region; edge of zone of regenerating gut at top of field. 675 ×.

FIGURE 9. Light microscope photograph of same field as Figure 8. Note the absence of acid phosphatase activity in all the neoblasts except those near the "active" region. a, zone of regenerating gut; b, inactive zone. 675 ×.

FIGURE 10. Gradient of acid phosphatase activity in neoblasts migrating (from right to left) to region of regenerating gut. a and b as in Figure 9. 675 ×.

the gut diverticuli were densely infiltrated by neoblasts in which the level of acid phosphatase activity was beginning to decline (Fig. 3). Coincident with the changes in the gut region, high levels of acid phosphatase activity were observed in the mucous glands and ventrolateral slime tracts and in the neoblasts in the pharynx.

The ingestion of cooked food was followed by the appearance of alkaline phosphatase activity in the forming food vacuoles (Fig. 4). After reaching a peak one to two days after food ingestion, the enzyme activity diminished somewhat more slowly than did that of acid phosphatase and was absent by the eighth day. Alkaline phosphatase activity in nerve fibers, mucous glands and pharynx was not affected by feeding and was undiminished after periods of starvation as long as 60 days (Fig. 5).

Preliminary studies have been made on phosphatase changes associated with regeneration following transection. On the sixth day after transection the regenerating pharynx was heavily infiltrated with neoblasts which had a moderately high level of acid phosphatase activity (Figs. 6 and 7). Figure 6 also shows an infiltration of both the original and the regenerating gut with neoblasts rich in acid phosphatase, suggesting that these cells were participating both in the degradation of the original gut (starvation effect) and in the reconstitution of the gut in the regenerating portion of the worm. The neoblasts at some distance from the sites of organ destruction or reconstitution ("resting" neoblasts) were lacking in acid phosphatase activity. As they migrated toward these sites they appeared to acquire progressively increasing amounts of acid phosphatase (Figs. 8–10).

The regenerating pharynx was also rich in alkaline phosphatase, predominantly localized in the neoblasts (Figs. 11 and 12).

Discussion

The changes in acid and alkaline phosphatase activities associated with feeding, starvation and regeneration in planarians suggest that these enzymes are intimately involved in the processes of nutrition, growth and repair. Little is known, however, about the stimuli to enzyme induction and the exact chemical reactions which are catalyzed by these enzymes *in vivo*.

Rosenbaum and Rolon (1960) reported the appearance of acid phosphatase activity in the gastrodermal cells of *Dugesia dorotocephala* and *Dugesia tigrina* within five minutes after the feeding of cooked liver, and they identified this with the formation of food vacuoles. Maximum acid phosphatase activity was present in all the food vacuoles 24 to 48 hours after feeding; the level of enzyme activity began to decline three days after feeding and was absent one week after feeding. Similar changes were observed in aminopeptidase and β-glucuronidase activities, although intracellular localization of the latter enzyme was not achieved. Müller, Toth and Törö (1962) described a similar relation of acid phosphatase and nonspecific esterase activity to the food vacuole cycle in *Amoeba proteus*. In unfed amebae, fine granules giving an intense acid phosphatase reaction (lysosomes?)

FIGURE 11. Regenerating pharynx 6 days after transection. The infiltrating neoblasts are rich in alkaline phosphatase. 75 ×.

FIGURE 12. Higher magnification of regenerating pharynx in Figure 11, showing alkaline phosphatase in neoblasts. 675 ×.

were distributed at random. Recently-formed food vacuoles showed only moderate enzyme activity but strongly staining granules were observed around these early vacuoles, often forming large aggregates. The authors suggest that these granules may be "enzyme carriers." Vacuoles containing still living or dying prey (*Tetrahymena pyriformis*) showed no demonstrable increase in acid phosphatase activity ("ingestion vacuoles"), but a sharp rise in enzyme activity occurred when the food organisms had been killed and most of the water had disappeared from the vacuoles ("digestion vacuoles"). Parallel changes occurred in non-specific esterase activity. The authors relate the increased activities of the two enzymes to the processes of intracellular digestion of food and suggest that ameba digestion vacuoles can be considered as lysosomes. Presumably they would be representatives of the type called "lysophagosomes" by de Duve (1961).

Our results confirm the recent report of Jennings (1962b) of a sequential appearance of acid and alkaline phosphatase activities in the gastrodermal cells of planarians following food ingestion. The apparently greater intensity and longer persistence of acid phosphatase activity in our preparations is probably due to better enzyme preservation in frozen sections than in paraffin-embedded material. There is general agreement that acid phosphatase and other acid hydrolases are involved in the initial stages of digestion in food vacuoles (Rosenbaum and Rolon, 1960; Müller *et al.*, 1962; Jennings, 1962b) but the function of the alkaline phosphatase which appears some hours after the beginning of digestion is unknown. Jennings (1962b) suggests that it "may be concerned in the release of energy needed for secretion of the various enzymes and the absorption of the products of digestion from the vacuoles." In studies on the rhynchocoelan, *Lineus ruber,* Jennings (1962a) reported the appearance of intense alkaline phosphatase activity in the luminal margins of the gut cells immediately after feeding, and he postulated a role of the enzyme in the process of phagocytosis of food. No such immediate response of alkaline phosphatase to food ingestion has been observed in planarians.

Prolonged starvation in planarians is characterized by the resorption of certain structures, notably the digestive and reproductive systems, and by the persistence of other more immediately essential structures, such as the pharynx, nervous system, protonephridia and lateral mucous glands. Survival for periods in excess of 60 days without food has been observed, and interesting changes in phosphatase activity occur during starvation. The earliest of these changes is a striking increase in acid phosphatase activity in the neoblasts, especially those surrounding the gut region, which is clearly in evidence on the fourth day following feeding. In the succeeding days the neoblasts infiltrate the gut where their hydrolytic enzymes presumably participate in the autolytic degradation of the gastrodermal cells in the later stages of starvation.

The sequence of changes in alkaline phosphatase activity during starvation is quite different. Following the decline in activity in the gastrodermal cells, leading to disappearance of the enzyme about eight days following feeding, there is no recurrence as in the case of acid phosphatase. However, the alkaline phosphatase activity of those structures which do not undergo resorption during starvation seems to be unaffected by feeding and starvation. Thus, even after 60 days starvation, there is intense alkaline phosphatase activity in the pharynx, the nerve fibers and the lateral mucous glands, *i.e.,* those structures which by preserving the capacity for locomotion and for ingestion of food, favor the survival of the organism.

The preliminary observations on regenerating planarians suggest that the neoblasts play a role both in the (probably) degenerative changes in the gut of the original segment and in the reconstitution of organs in the regenerating segment. In both cases the neoblasts in these sites have a high level of acid phosphatase activity. Since the "resting" neoblasts show no acid phosphatase activity (Figs. 8–10), this must represent enzyme induction and a participation of acid phosphatase in both degradative and regenerative processes. The neoblasts in the sites of organ regeneration have intense alkaline phosphatase activity also, but the functional significance of this observation is uncertain, since resting neoblasts also show alkaline phosphatase activity (Pedersen, 1959).

The results of the present study implicate acid phosphatase in a variety of degradative processes, presumably autolytic in nature, all of which results in the conversion of endogenous or exogenous compounds into building materials for maintenance and repair. An apparent discrepancy between our results and those of earlier studies on the histological changes during starvation in planarians requires some comment. Willier *et al.* (1925) reported that the cells of the intestinal epithelium show practically no change until after six weeks of starvation, when they begin to undergo degeneration with reduction in size. Our observation of intense acid phosphatase activity in the region of the intestinal epithelial cells after much shorter periods of starvation suggests that the enzymatic phase of autolytic degradation may begin much earlier, but that this does not lead immediately to histologically demonstrable degeneration of the cells. The participation of acid phosphatase in the reconstitution of organs in regenerating planarians suggests the simultaneous occurrence of degradative and synthetic reactions, the one perhaps providing the raw materials for the other. The functional significance of the alkaline phosphatase changes is unknown. Its presence in the neoblasts in regions of regeneration may reflect a participation in organogenesis (Junqueira, 1950; Vorbrodt, 1958), but this is conjectural in view of the occurrence of alkaline phosphatase in resting neoblasts.

The senior author is indebted to Dr. E. Ruffin Jones, Jr., Biology Department, University of Florida, for aid in earlier studies (unpublished) on the hydrolytic enzymes of planaria.

SUMMARY

1. The ingestion of cooked food by starved planarians is followed by the appearance of high levels of acid, and later of alkaline phosphatase activities in the gastrodermal cells. The enzyme activities decline gradually, to disappear after 7–10 days. Beginning about 4 days after feeding, the gut region is progressively invaded by neoblasts with high acid phosphatase activity. Intense alkaline phosphatase activity persists in certain "essential" structures (nerve fibers, gland cells, protonephridia) even after periods of starvation as long as 60 days.

2. During regeneration following transection, neoblasts rich in acid phosphatase invade both the regenerating organs and the degenerating structures of the original

segment. This represents enzyme induction, since resting neoblasts show no acid phosphatase activity. Neoblasts with alkaline phosphatase activity are abundant in the regions of regeneration, but the significance of this observation is uncertain since alkaline phosphatase activity also characterizes resting neoblasts.

3. It is suggested that the lysosomal acid hydrolases (typified by acid phosphatase) are involved not only in the early stages of digestion in the food vacuoles, but also in the autolysis of dispensable organs during starvation and in the tissue breakdown which precedes regeneration in transected planarians.

LITERATURE CITED

Danielli, J. F., and C. F. A. Pantin, 1950. Alkaline phosphatase in protonephridia of terrestial nemertines and planarians. *Quart. J. Micr. Sci.*, **91**: 209–214.

de Duve, C., 1961. *In:* Biological Approaches to Cancer Chemotherapy, New York, Academic Press, Inc., pp. 101–112.

Gazso, L. R., L. J. Török and Gy. Rappay, 1961. Contributions to the histochemistry of the nervous system of planarians. *Acta Biol. Acad. Sci. Hung.*, **11**: 411–428.

Gomori, G., 1952. Microscopic Histochemistry. University of Chicago Press, Chicago, Illinois.

Jennings, J. B., 1962a. A histochemical study of digestion and digestive enzymes in the rhynchocoelan *Lineus ruber* (O. F. Müller). *Biol. Bull.*, **122**: 63–72.

Jennings, J. B., 1962b. Further studies on feeding and digestion in triclad Turbellaria. *Biol. Bull.*, **123**: 571–581.

Junqueira, L. C. U., 1950. Alkaline and acid phosphatase distribution in normal and regenerating tadpole tails. *J. Anat.*, **84**: 369–373.

Müller, M., and I. Törö, 1962. Studies on feeding and digestion in protozoa. III. Acid phosphatase activity in food vacuoles of *Paramecium multimicronucleatum. J. Protozool.*, **9**: 98–102.

Müller, M., J. Toth and I. Törö, 1962. Studies on feeding and digestion in protozoa. IV. Acid phosphatase and nonspecific esterase activity of food vacuoles in *Amoeba proteus. Acta Biologica Acad. Sci. Hung.*, **13**: 105–116.

Pedersen, K. J., 1959. Cytological studies on the planarian neoblast. *Zeitschr. f. Zellforsch.*, **50**: 799–817.

Rosenbaum, R. M., and C. I. Rolon, 1960. Intracellular digestion in the phagocytic cells of planaria. *Biol. Bull.*, **118**: 315–323.

Vorbrodt, A., 1958. Histochemically demonstrable phosphatase and protein synthesis. *Exp. Cell Res.*, **15**: 1–20.

Willier, B. H., L. H. Hyman and S. A. Rifenburgh, 1925. A histochemical study of intracellular digestion in triclad flatworms. *J. Morphol. and Physiol.*, **40**: 299–340.

STUDIES ON THE HEXAPOD NERVOUS SYSTEM. VI. VENTRAL NERVE CORD SHORTENING; A METAMORPHIC PROCESS IN GALLERIA MELLONELLA (L.) (LEPIDOPTERA, PYRALLIDAE)[1]

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One of many spectacular occurrences during the metamorphosis of holometabolous insects is the transformation of the larval central nervous system into that of the adult. An account of the gross features of the change as it occurs in certain Lepidoptera was given in the last century (Newport, 1832, 1834). The salient characteristics noted include: a concentration of the mesothoracic, metathoracic, and first two abdominal ganglia; a shortening of connectives between the brain and subesophageal ganglion, and between the prothoracic and mesothoracic ganglia. In *Papilio urticae* L., under the ambient temperatures employed, these changes were realized during the first 58 hours after pupation. This contrasted with observations on *Sphinx ligustri* L., where nearly six months of developmental arrest (diapause) intervene between the most active phases of the phenomenon.

Brandt's (1879) extensive comparative study partly confirmed Newport's findings, but called attention to departures from the above pattern of reorganization in different lepidopteran species. He also depicted a fusion of the last three abdominal ganglia, an event undescribed by Newport.

The prospect of using the greater wax moth, *Galleria mellonella* (L.), in an extended analysis of neurometamorphosis has prompted the following investigation of the shortening process. Concomitant cytological features will be reported in a future communication.

METHODS

Stock cultures of *Galleria* were maintained by placing several adult males and females in a screen-topped gallon jar containing a larval diet mixture of 1200 ml. Gerber Mixed Cereal, 100 ml. honey, 100 ml. glycerin, and 50 ml. water. These were kept in darkness in a constant temperature cabinet (32–35° C.). Each culture was divided at least once and replenished with fresh diet to circumvent undesired effects of crowding. Last-instar larvae were gathered from cocoons spun along the sides of the containers.

To ascertain deviations in nerve cord length due to variations in body size, last-instar larvae were separated into two groups. Individuals of one group weighed 170–190 mg., those of the other, 200–250 mg. Because values obtained from the two groups were in reasonable accord, they have been combined for statistical treatment.

Ten to 15 larvae were routinely placed in a 105 × 20 mm. plastic Petri-type culture dish, provided with cardboard tents, and maintained at a uniform tempera-

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[1] This study was supported by National Institutes of Health Grant No. B-3845.

ture (30–33° C.). By the end of a day the majority had enclosed themselves within cocoons beneath the tents. The ends of each cocoon were opened at 8–12-hour intervals to permit inspection for signs of impending pupation, which are described below. Larvae demonstrating such signs were removed from their cocoons, and placed in separate dishes also kept at 30–33° C. The time during which they cast their exuviae, thus revealing "white" (untanned) pupae, was known precisely, or else approximated to within three hours.

Ventral nerve cords in desired stages of development were exposed by dorsal dissections of living insects pinned to the bottoms of Syracuse watch glasses partly filled with paraffin. The animals were immersed in Yeager's (1939) insect magnesium saline during the operation. Interganglionic connectives were measured to the nearest 0.1 mm. at a magnification of 25 diameters by means of an ocular micrometer inserted in a dissecting microscope (Wild-Greenough). Particular care was taken to minimize stretching the cords beyond their rest lengths. Measurements were routinely completely 40–50 minutes after dissection was initiated. Comparative measurements made on the same connectives at the beginning and end of this period usually differed by less than 10%.

Measurements of nerve cords from pupae and adults reared from last-instar larvae kept at 30–33° C. and 70% relative humidity (R. H.) over KOH (Buxton, 1931) did not differ significantly from those made on individuals of comparable developmental stage maintained at the same temperature, but at ambient R. H. Consequently, no attempt was made to control this factor.

Developmental Stages

All systems of a multicellular organism do not complete differentiation simultaneously. This, and the fact that developmental transitions are gradual, rather than saltatory, necessitates that selection and definition of stages be arbitrary. One criterion which might be used is the ability of the larva to spin a cocoon. As indicated by Piepho (1950) and Wiedbrauck (1955) this appears to be under hormonal control, a decrease in juvenile hormone concentration, such as occurs at the outset of pupation (Gilbert and Schneiderman, 1961; Williams, 1961), resulting in cessation of spinning.

During the present investigation an event which precedes total loss of spinning activity was detected. This involves the ability of last-instar larvae to protract and retract their anal legs, or postpedes, when these are touched. Because the reflex is lost 19–27 hours prior to ecdysis, it has served as a useful external sign of incipient pupation. The change does not seem to interfere with locomotion, nor with the larva's ability to right itself when placed in a supine position.

Last-instar larvae which have constructed cocoons, which can move their postpedes, and which can re-spin shall be referred to as stage I larvae. Stage II larvae lack the ability to protract and retract their postpedes. At the outset of this stage they can locomote readily, but this capability is soon lost. Spinning activity also ceases during this period. Stage III insects cannot walk, nor can they right themselves. They twist their abdomens vigorously from side to side, an activity which aids in shedding the larval exuviae. Stage III includes the "pharate" ("cloaked") pupal stage (Hinton, 1958). It terminates at ecdysis with the uncovering of the "white" pupa.

TABLE 1—*Mean lengths and standard deviations (millimeters) of ventral nerve cord segments in Galleria mellonella (L.) during progressive developmental stages*

Stage or Hours	SE–I	I–II	II–III	III–1	1–2	2–3	3–4	4–5	5–6	6–7, 8	6+7, 8	II+III +1+2
Stage I	0.1 ±0.05	1.1 ±0.11	1.4 ±0.10	0.4 ±0.06	1.1 ±0.10	1.2 ±0.09	1.4 ±0.11	1.4 ±0.11	1.4 ±0.12	0.9 ±0.09	1.8 ±0.15	4.3 ±0.22
Number	30	30	30	30	30	30	30	30	30	30	20	20
Stage II	0.1 ±0.05	1.0 ±0.09	1.2 ±0.14	0.4 ±0.08	0.9 ±0.12	1.2 ±0.08	1.4 ±0.10	1.4 ±0.11	1.3 ±0.07	0.8 ±0.1	1.6 ±0.14	3.7 ±0.31
Number	9	9	9	9	9	9	9	9	9	9	9	9
Stage III	0.1 ±0.05	0.8 ±0.10	1.0 ±0.13	—	0.7 ±0.17	1.1 ±0.07	1.3 ±0.07	1.3 ±0.08	1.3 ±0.09	0.6 ±0.06	1.5 ±0.08	3.2 ±0.28
Number	11	10	11		11	11	11	10	10	10	10	11
0–5	0.1 ±0.05	0.7 ±0.09	0.8 ±0.16	—	—	0.9 ±0.10	1.2 ±0.11	1.3 ±0.12	1.3 ±0.11	0.6 ±0.10	1.4 ±0.14	2.7 ±0.20
Number	11	11	11	—	—	11	11	11	11	11	11	11
6–11	0.1 ±0.05	0.6 ±0.07	0.7 ±0.09	—	—	0.9 ±0.10	1.1 ±0.09	1.3 ±0.11	1.3 ±0.08	0.4 ±0.12	1.1 ±0.16	2.4 ±0.15
Number	13	13	13	—	—	13	13	13	13	12	11	11
12–17	0.2 ±0.05	0.5 ±0.04	0.6 ±0.09	—	—	0.8 ±0.16 *1.3 ±0.06	1.1 ±0.11	1.3 ±0.08	1.2 ±0.18	—	1.0 ±0.10	2.6 ±0.19 **2.0 ±0.11
Number	13	12	13	—	—	9 *13	13	13	13	—	13	11 **7
18–23	0.3 ±0.07	0.4 ±0.06	0.5 ±0.1	—	—	0.7 ±0.11 *1.4 ±0.13	1.1 ±0.09	1.2 ±0.13	1.2 ±0.12	—	0.9 ±0.14	2.3 ±0.18 **1.7 ±0.12
Number	10	11	11	—	—	8 *10	11	11	11	—	10	8 **9
24–29	0.3 ±0.08	0.4 ±0.08	0.4 ±0.07	—	—	0.8 ±0.11 *1.5 ±0.19	1.1 ±0.10	1.2 ±0.08	1.2 ±0.13	—	0.8 ±0.18	2.3 ±0.08 **1.5 ±0.15
Number	12	12	11	—	—	6 *11	11	12	11	—	11	11 **4
30–35	0.5 ±0.05	0.4 ±0.09	—	—	—	*1.8 ±0.16	1.2 ±0.08	1.4 ±0.08	1.3 ±0.10	—	0.6 ±0.03	**1.2 ±0.09
Number	11	12	—	—	—	*11	11	11	11	—	12	**12
36–41	0.4 ±0.06	0.3 ±0.07	—	—	—	*2.1 ±0.11	1.2 ±0.09	1.3 ±0.13	1.4 ±0.18	—	0.5 ±0.05	**1.1 ±0.08
Number	11	11	—	—	—	*12	11	10	10	—	8	**9
42–47	0.5 ±0.08	0.3 ±0.07	0	—	—	*2.2 ±0.18	1.1 ±0.10	1.3 ±0.13	1.4 ±0.14	—	0.6 ±0.05	**1.0 ±0.07
Number	10	10	10	—	—	*10	10	10	10	—	10	**10
48–53	0.5 ±0.05	0.3 ±0.06	0	—	—	*2.3 ±0.08	1.1 ±0.07	1.2 ±0.10	1.4 ±0.10	—	0.5 ±0.07	**1.0 ±0.04
Number	9	9	9	—	—	*9	8	9	9	—	9	**9
Adult ♀ <1 day old	0.8 ±0.09	0.4 ±0.05	0	—	—	*2.7 ±0.13	1.3 ±0.14	1.6 ±0.10	2.0 ±0.26	—	0.6 ±0.03	**1.0 ±0.06
Number	12	12	12	—	—	*12	12	12	12	—	12	**12
Adult ♂ <1 day old	0.8 ±0.09	0.5 ±0.15	0	—	—	*2.8 ±0.14	1.2 ±0.11	1.3 ±0.11	1.4 ±0.16	—	0.5 ±0.04	**0.9 ±0.05
Number	11	12	12	—	—	*12	12	12	12	—	12	**12

* Posterior border of coalescing white mass to anterior edge of third abdominal ganglion. ** Anterior border of mesothoracic ganglion to posterior edge of coalescing white mass.

Stage of pupal development was defined by the number of hours after ecdysis. Pupal age-class intervals of five hours were used throughout (Table I). An intermediate time for each interval is designated on the abscissas of Figures 2 and 3. Thus, individuals in the age class 0–5 hours are considered 3 hours old; those in the age class 6–11 hours, 9 hours old; etc. Stage II precedes ecdysis by 19–27 hours (25 of 33 cases recorded); stage III by 6–15 hours (18 of 23 cases recorded).

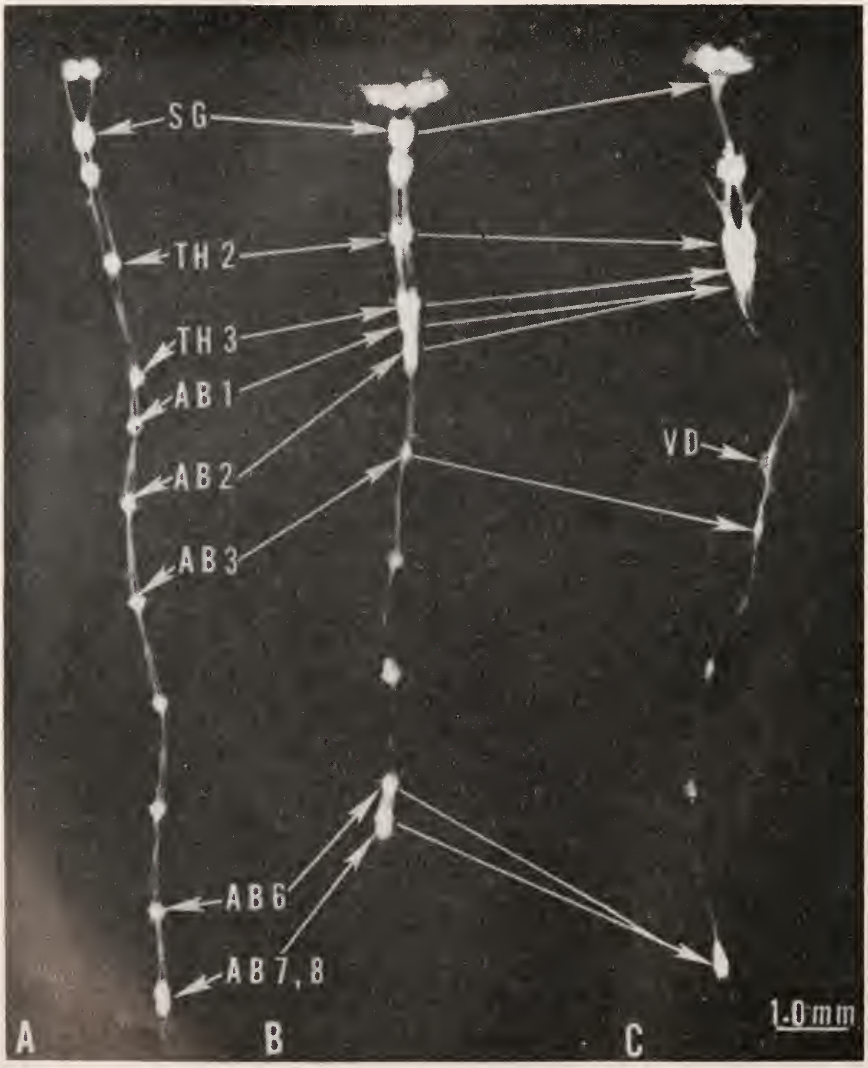

FIGURE 1. Dorsal view of central nervous systems of *Galleria*. Fixed *in situ* with 95% ethanol, removed, and preserved in 10% formalin. (A) Stage I larva; (B) Pupa, 12–15 hours after ecdysis; (C) Adult female. AB1, AB2, AB3, AB6, AB7,8, Abdominal ganglia; SG, Subesophageal ganglion; TH2, TH3, Thoracic ganglia; VD, Ventral diaphragm.

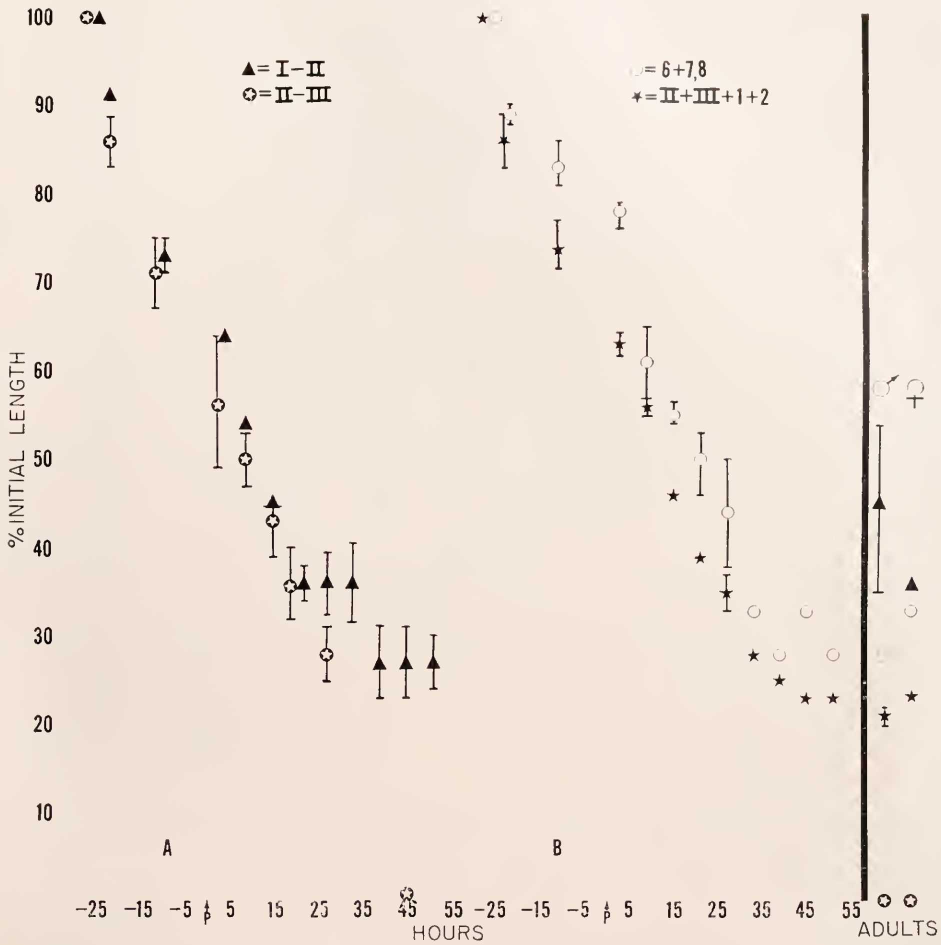

Figure 2. Per cent of mean initial (stage I) lengths remaining at progressive developmental stages. Standard deviations indicated by vertical lines, except where obscured by a symbol. (A) I–II, connectives between first and second thoracic ganglia; II–III, connectives between the second and third thoracic ganglia; (B) 6 + 7,8, length from anterior border of abdominal ganglion six to posterior border of abdominal ganglion eight; II + III + 1 + 2, length from anterior border of mesothoracic ganglion to the posterior border of the second abdominal ganglion. P, time of ecdysis. Percentages of stage I lengths remaining in adult males and females included at the extreme right.

Accordingly, data for stage II insects are plotted at −23 hours; those for stage III at −11 hours. Data for stage I are plotted at −26 hours, regardless of the fact that many of these insects were probably further removed from ecdysis than this.

Pattern of Reorganization

The ventral nerve cord of a stage I larva (Fig. 1A) consists of twelve definitive ganglia, all fairly uniform in size. The first eleven are associated by

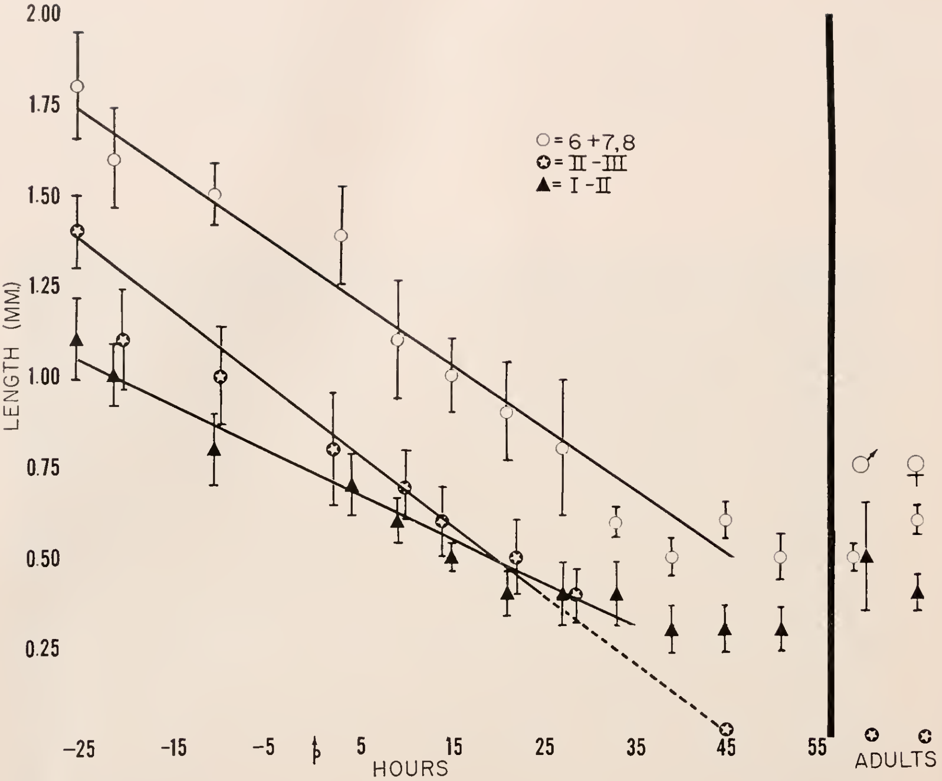

Figure 3. Mean lengths of selected segments of the ventral nerve cord during progressive developmental stages. Values for adult males and females are included at the extreme right. Standard deviations indicated by vertical lines. Symbols as in Figure 2.

paired connectives which vary in length. These are visible at low magnifications. The seventh and eighth abdominal ganglia, however, are contiguous. The ventral nerve cord is associated with the brain by a pair of circumesophageal connectives.

Comparison with the ventral nerve cord of a newly emerged adult (Fig. 1C) reveals prominent differences. The adult possesses nine externally recognizable ventral ganglia; the first, second, and sixth abdominal ganglia of the larva have vanished. The paired nature of the connectives between the subesophageal and first thoracic ganglia and between abdominal ganglia is no longer distinct. The second and third thoracic ganglia, lacking intervening connectives, are now contiguous. The connectives between the subesophageal ganglion and brain, and between the first and second thoracic ganglia, have also shortened; those between the subesophageal and first thoracic ganglia have lengthened. There is a striking disparity in comparative volumes of thoracic and abdominal ganglia, a reflection of disharmonic growth along the cranio-caudal axis. Another difference is that the adult abdominal interganglionic connectives are attached to the ventral diaphragm (VD, Fig. 1C), which, in turn, inserts on the abdominal exoskeleton. Contractions of muscle fibers in the diaphragm cause the abdominal nerve cord to lash from side to side within the hemocoele.

Sequential measurements of the connectives located between the first and second (I–II) and second and third (II–III) thoracic ganglia indicate that they lose 35–45% of their mean initial lengths before ecdysis (Fig. 2A). By 30–40 hours after ecdysis connectives I–II shorten maximally, losing about 75% of their original length. This contrasts with connectives II–III which continue to diminish until *ca.* 45 hours, when they disappear, leaving the ganglia contiguous.

It might be suspected that decrease in the length of I–II is not due to shortening, but is simply a result of overgrowth by the first and second thoracic ganglia. Such an interpretation is not supported by comparative measurements. These show that no more than half the 0.6–0.7 mm. lost could possibly be accounted for in this manner, and then only if all growth were directed toward I–II.

Shortening of I–II and lengthening of connectives between the subesophageal and first thoracic ganglia (SE–I) are not in phase. By 12–17 hours after ecdysis, when elongation of SE–I is first detectable, shortening of I–II is approximately 75% complete (Table I). Partial temporal separation of these two morphogenic processes occurs elsewhere within the ventral nerve cord, and shall be mentioned again below.

Reorganization, which results in disappearance of the first, second, and sixth abdominal ganglia, is indicated by Figure 1B. The first and second abdominal ganglia approach the third thoracic anteriorly, while the sixth meets the contiguous seventh and eighth posteriorly. During shortening the intervening connectives increase in diameter until they are nearly as wide as the ganglia which are about to coalesce. The discreteness of the connectives involved is lost before shortening is concluded. This has necessitated measuring from the anterior border of one ganglion to the posterior border of another. Shortening which results in concentration of the first two abdominal and the second and third thoracic ganglia was approximated by measuring from the anterior border of the second thoracic ganglion to the posterior border of the second abdominal ganglion (II + III + 1 + 2, Table I; Fig. 2B). Shortening, which results in coalescence of abdominal ganglia six, seven, and eight, was followed by measuring from the anterior border of six to the posterior border of eight (6 + 7,8 Table I; Figs. 2B; 3).

The entire second abdominal ganglion does not coalesce with the fused metathoracic-first abdominal complex. Instead, anterior migration of the definitive second abdominal ganglion ceases 12–17 hours following ecdysis (Fig. 1B). After that time an opaque white mass, presumably consisting of internal components of the second abdominal ganglion, continues forward. As this proceeds the second abdominal ganglion diminishes until an inconspicuous "hull" remains. The latter subsequently moves back as connectives between it and the advancing white mass elongate. The "hull" disappears 30–35 hours after ecdysis. Shortening, as determined by measuring II + III + 1 + 2 (Table I), is about 70% complete 12–17 hours after ecdysis, when elongation of conectives between the "hull" and white mass first becomes apparent.

Abdominal interganglionic connectives 2–3, 3–4, 4–5, and 5–6 do not shorten appreciably. Application of the *t*-test for significance between the highest and lowest means obtained for each during metamorphosis yielded *t*-values of 0.08, 0.16, 0.11, and 0.10, respectively.

Events resulting in coalescence of the sixth abdominal ganglion with seven and eight resemble those noted during fusion of the first two abdominal ganglia with the third thoracic. Thus, it appears that the contents, but not the "hull" of ganglion 6 become incorporated with 7,8. As connectives 6–7,8 shorten they increase in diameter, and it becomes increasingly difficult to separate boundaries of the ganglia (Fig. 1B). Consecutive measurements of 6 + 7,8 reveal that coalescence is completed 30–40 hours after ecdysis (Figs. 2B, 3).

When the mean lengths of selected segments of the ventral nerve cord are plotted against developmental time, the distributions depicted in Figure 3 are obtained. The origins and slopes of three of these were estimated by assuming linearity, and by applying the method of least squares. Extent of shortening per 24 hours approximated in this manner is 0.27 mm. for connectives I–II ($Y = 1.024 + 0.0112X$); 0.46 mm. for II–III ($Y = 1.37 + 0.0192X$); and 0.41 mm. for 6 + 7,8 ($Y = 1.73 + 0.0171X$). Tests for homogeneity of regression on each of the three combinations of the three lines were significant in each case (I–II vs. II–III, $t = 11.56^{***}$; I–II vs. 6 + 7,8, $t = 9.96^{***}$; and II–III vs. 6 + 7,8, $t = 2.38^{*}$).

DISCUSSION

The consecutive measurements made during the course of this study clearly demonstrate that ganglionic concentration is accomplished by shortening of intervening connectives. The explanation proposed by Murray and Tiegs (1935) for ganglionic concentration in the beetle, *Calandra oryzae*, namely, that it is due to a "proliferation of cells" and subsequent overgrowth, cannot be accepted for *Galleria*. The present study does not indicate which of the nerve cord components is responsible for decrease in length of the connectives, but the conclusion that it is caused by extraneuronal cellular migration, neuron shortening, or both, seems unavoidable.

The extent to which different sectors of the ventral nerve cord shorten is variable. The connectives between the second and third, third and fourth, fourth and fifth, and fifth and sixth abdominal ganglia do not shorten significantly. Connectives between the first and second thoracic ganglia shorten approximately 75% of their mean initial length, while those between the second and third disappear. Connectives between the third thoracic and first abdominal ganglia, first abdominal and second abdominal ganglia, and abdominal ganglia 6 and 7,8 not only disappear, but their associated ganglia coalesce. Thus, the following gradations prevail: (1) No significant shortening. (2) Partial shortening. (3) Complete shortening with establishment of contiguity between centers. (4) Complete shortening with coalescence of centers.

Gross features predict extensive concomitant modifications at the tissue and cellular level during shortening. The composition and fate of the ganglionic constituents of the advancing white mass and the shortening connectives proper are but a few of the manifestations which require histological and cytological clarification.

Ventral nerve cord shortening, well under way before ecdysis, is concluded 30–45 hours after. The gross features of neurometamorphosis described here require approximately three days for completion, or approximately 33% the mean total time from onset of pupation (stage II) to adult emergence.

Much of the shortening which occurs is compensated by subsequent elongation. Extent of shortening of connectives I–II and extent of elongation of connectives between the subesophageal and first thoracic ganglia are nearly identical (Table I). Little, if any, decrease in total ventral nerve cord length results. Similarly, loss of interganglionic connectives between the third thoracic and first abdominal ganglia, and between the first and second abdominal ganglia is entirely compensated by elongation between the second and third abdominal ganglia.

The resultant effect is an adult ventral nerve cord only slightly shorter than that of the stage I larva from which it has developed. Of greater significance, perhaps, is the correlation between the gross structural reorganization of the ventral nerve cord and the skeleto-muscular system. During metamorphosis the thoracic and first two abdominal metameres are brought closer together, the prothoracic, metathoracic, and first two abdominal segments suffering extensive reduction during the process. Shortening of the corresponding interganglionic connectives is in accord with these changes.

Statistical analysis of these data is principally due to the generous efforts of Dr. Howell V. Daly. I thank Dr. Roderick Craig and Dr. Howell V. Daly for critically reading the manuscript, and for relevant suggestions. The technical assistance of Mrs. Nancy Luykx is also gratefully acknowledged.

SUMMARY

1. Gross features of interganglionic connective shortening during metamorphosis of *Galleria mellonella* (L.) are described. Gradations range from no significant shortening, to partial shortening, to shortening with establishment of contiguity between ganglia, to complete shortening with coalescence of ganglia.

2. Under the experimental conditions employed, shortening commences about a day prior to ecdysis, and is completed 30–45 hours after ecdysis. The rates at which various connectives shorten differ significantly from one another. If linearity is assumed, these range from 0.3 to 0.5 mm. per day.

3. Much of the shortening is compensated by subsequent elongation of connectives. The two morphogenic processes are not in phase; shortening is 70–75% complete before elongation can be detected.

4. The adult ventral nerve cord is about 15–20% shorter than that of the stage I larva from which it has developed. Shortening has altered the relative locations of certain of the ganglia so that they are in accord with structural reorganization of the skeleto-muscular system. Not only are ganglia retained close to their effector organs as a consequence, but conduction times between certain of the centers would be expected to be reduced.

LITERATURE CITED

Brandt, E., 1879. Vergleichend-anatomische Untersuchungen über das Nervensystem der Lepidopteren. *Horae Soc. Entomol. Ross.*, **15**: 1–16.

Buxton, P. A., 1931. The measurement and control of atmospheric humidity in entomological problems. *Bull. Ent. Res.*, **22**: 431–457.

Gilbert, L. I., and H. A. Schneiderman, 1961. The content of juvenile hormone and lipid in Lepidoptera: sexual differences and developmental changes. *Gen. Comp. Endocrin.* **1**: 453–472.

HINTON, H. E., 1958. Concealed phases in the metamorphosis of insects. *Sci. Prog.*, **182**: 260–275.

MURRAY, F. V., AND O. W. TIEGS, 1935. The metamorphosis of *Calandra oryzae*. *Quart. J. Micr. Sci.*, **77**: 405–495.

NEWPORT, G., 1832. On the nervous system of the *Sphinx ligustri*, Linn., and on the changes which it undergoes during a part of the metamorphosis of the insect. *Phil. Trans. Roy. Soc. London*, **122**: 383–398.

NEWPORT, G., 1834. On the nervous system of the *Sphinx ligustri*, Linn., (Part II) during the latter stages of its pupa and its imago state; and on the means by which its development is effected. *Phil. Trans. Roy. Soc. London*, **124**: 389–423.

PIEPHO, H., 1950. Hormonale Grundlagen der Spinntätigkeit bei Schmetterlingsraupen. *Zeitschr. Tierpsychol.*, **7**: 424–434.

WIEDBRAUCK, J., 1955. Vom Spinnen bei Schmetterlingsraupen und seiner Abhängigkeit von Metamorphosehormonen. *Zeitschr. Tierpsychol.*, **12**: 176–202.

WILLIAMS, C. M., 1961. The juvenile hormone. II. Its role in the endocrine control of molting, pupation, and adult development in the Cecropia silkworm. *Biol. Bull.*, **121**: 572–585.

YEAGER, J. F., 1939. Electrical stimulation of isolated heart preparations from *Periplaneta americana*. *J. Agr. Res.*, **59**: 121–137.

X-IRRADIATION–INDUCED CONGENITAL ANOMALIES IN HYBRID MICE[1]

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Heterosis, or hybrid vigor, in radiobiological studies, has been demonstrated for the adult mouse (Rugh and Wolff, 1958); for the testes (Rugh, Funk and Wohlfromm, 1961) and for some induced congenital anomalies (Rugh, Wohlfromm and Grupp, 1961). It was further shown (Grahn, 1958) that there are non-additive (heterotic) effects when a sensitive strain is crossed with a particularly resistant strain (C57BL/6 × BALB/c), while the variance in sensitivity, as measured by the dosage-mortality slope, appears to be merely additive. It was decided to carry the experiment one further generation, by crossing the hybrids with themselves as well as with each of the parental strains, in order to detect, if possible, any influence caused by the preponderance of either genotype. The test was made by x-irradiating the embryos at 8.5 days' gestation and determining the effect at 18.5 days, just prior to the expected delivery.

MATERIALS AND METHOD

The mice used were the CF1 Swiss white strain from Carworth Farms and the C57 BL/6 Blacks from the Bar Harbor Laboratory. These strains are easily interbred and give viable offspring. All matings were made overnight and the females with vaginal plugs were separated the following morning and marked as 0.5 day pregnant. While this exposure of the females could give a range of conception of 16 hours in simultaneously exposed mice it is now known that most of the matings occur early in this period and that all of the mice were at least 0.5 day pregnant as of 9 A.M. the morning following the introduction of males. Since the x-irradiations were to occur at 8.5 days it is believed that the time range of conception was somewhat less significant than if the exposure were shortly after conception. The number of pregnancies for any combination was at least 21 so that the time variance in fertilization is averaged out.

Whole body x-irradiation was achieved with parallel tubes in cross-fire, each at 67 cm. distance from the gravid uterus (filtration 0.28 Cu and 0.50 Al, half value layer 0.6 Cu, dose rate 50 r/min., total dose 200 r). The machine was run at 184 KVP, and 30 MA. The mice were not anesthetized, but were placed in a plastic cage within which the dosimetric determinations were made.

Since mice often destroy or eat their offspring, when they are abnormal, it was necessary in this study to sacrifice the pregnant mice at 18.5 days (prior

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[1] Based in part on work performed under Contract AT (30-1)-2740 for the U. S. Atomic Energy Commission and aided by Grant RH 97 from Div. Radiological Health, Bur. State Services, Public Health Service.

to the expected delivery) and analyze the effects of x-irradiation at that time. It was also found that there was considerable variation between and within litters, even among the controls, so that to provide adequate statistical data, a minimum of 21 (and maximum of 42) pregnancies per set of categories was collected. The minimum number of implantations for any set of categories was 235 (and the maximum was 471), either figure giving a statistically adequate number so that anomaly percentages have significance. Each pregnant mouse was killed by cervical dislocation and the gravid uterus dissected out immediately. On the basis of prior studies, as well as new current findings, the categories included among the anomalous conditions were resorptions, dead fetuses, and obvious CNS, eye, visceral and tail anomalies. The "normals" included all which appeared grossly normal, but all or many of these could exhibit microscopic anomalies not readily apparent. It cannot be assumed that a "normal-appearing" fetus in a litter where there are gross anomalies caused by irradiation will itself be entirely normal. Thus, this paper categorizes only those gross anomalies which are readily identifiable, and classes as "normal" all fetuses which appear grossly to be normal.

EXPERIMENTAL DATA

The data are presented herein by means of tables which include all of the implantations examined, namely some 296 pregnancies and 2780 implantations. It can be seen readily that 200 r at 8.5 days affects the C57 embryo much more drastically than it does the CF1 embryo of the same age, reducing the "normals" to 0.4% and 40%, respectively. It had previously been shown that the first generation hybrids were more resistant to this x-irradiation insult than were either of these parent stocks (Haverland and Gowen, 1960; Rugh, Wohlfromm and Grupp, 1961; Nash and Gowen, 1962). However, when one obtains the F_2 by mating the first hybrid generation, it is found again that there is a higher radioresistance in the appearance of more "normals" (53%). This may be suggestive of heterosis. When the first generation hybrids are crossed with either of the original parental stocks, it is found that slight heterosis is still indicated when the parent genotype is CF1 but not when it is C57. However, even in this latter cross the results are more favorable than in the C57 × C57 cross, simply because of the presence of CF1 genes, and there are as many normals as there are in the control cross of CF1 × CF1. This means simply that either the influence of the CF1 genes, in preponderance, affords some radioresistance to the embryos or when the C57 genes are in preponderance the initial first generation heterosis is reduced (see comparable data of Nash and Gowen, 1962). However, in every cross tried, the original C57 genotype, enforced by any CF1 genes, proved to be at least as radioresistant as the F_1 of the CF1 × CF1 cross. This suggests a radioresistant influence comparable to dominance.

It is known that more eggs are ovulated than are fertilized, a fact based upon the number of corpora lutea and the number of implantations (Otis, 1953). The data of this paper begin with implantations. Among these are some that are destined to die and be resorbed, in both strains of mice. But since the percentage of resorptions in the control CF1 and the control C57 mice were about the same proportion, they are included in all calculations.

The so-called "normal" mice may not, in fact, be completely normal. They

TABLE I

X-irradiation-conditioned congenital anomalies in the embryos of hybrid mice

Mouse cross		Preg- nancies	Implanta- tions	"Normal"	Dead	CNS anomalies	Anemic—stunted	Eye defects	Eviscer- ation	Resorptions	Short tails
CF1×CF1 =	C	42	471	399 (85)	4 (0.85)	0	8 (1.7)	0	0	60 (13)	0
	X	21	235	94 (40)	29 (12)	33 (14)	1 (0.43)	2 (0.85)	0	76 (32)	0
C57×C57 =	C	33	253	203 (80)	6 (2.3)	3 (1.2)	3 (1.2)	6 (2.3)	1 (0.39)	31 (12)	0
	X	34	252	1 (0.4)	18 (7.1)	0	0	1 (0.40)	1 (0.40)	231 (92)	0
C57/CF1×C57/CF1 =	C	28	269	246 (91)	0	1 (0.37)	2 (0.74)	0	0	20 (7.4)	0
	X	28	248	132 (53)	39 (16)	7 (2.8)	9 (3.6)	13 (5.2)	2 (0.8)	46 (18.6)	28 (11.3)
C57/CF1×CF1 =	C	28	273	247 (90)	3 (1.1)	1 (0.37)	0	0	0	22 (8.1)	0
	X	27	264	122 (46)	37 (14)	20 (7.6)	16 (6.1)	11 (4.1)	0	58 (22)	7 (2.6)
C57 CF1×C57 =	C	28	261	238 (91)	1 (0.4)	1 (0.4)	1 (0.4)	0	0	20 (7.7)	0
	X	27	254	102 (40)	30 (11.8)	2 (0.8)	12 (4.7)	7 (2.8)	4 (1.6)	97 (38)	12 (4.7)
Totals		296	2780								

C = Controls
X = X-irradiated to 200 r at 8.5 days gestation (Percentages in parentheses)

were normal-appearing mice since they were the survivors of x-irradiations which conditioned anomalies in the same litter, but it is very doubtful that they themselves were normal. The "dead" were those which survived until the third trimester and had the distinguishable features of fetuses. Those which were anemic and/or stunted may have been moribund, but were alive at the time of analysis. The eye defects, normally seen in C57 stock, were expressed as microphthalmia, anophthalmia, etc. In a few cases evisceration was found, in which the developing visceral organs failed to be enclosed in overgrowth of the abdomen.

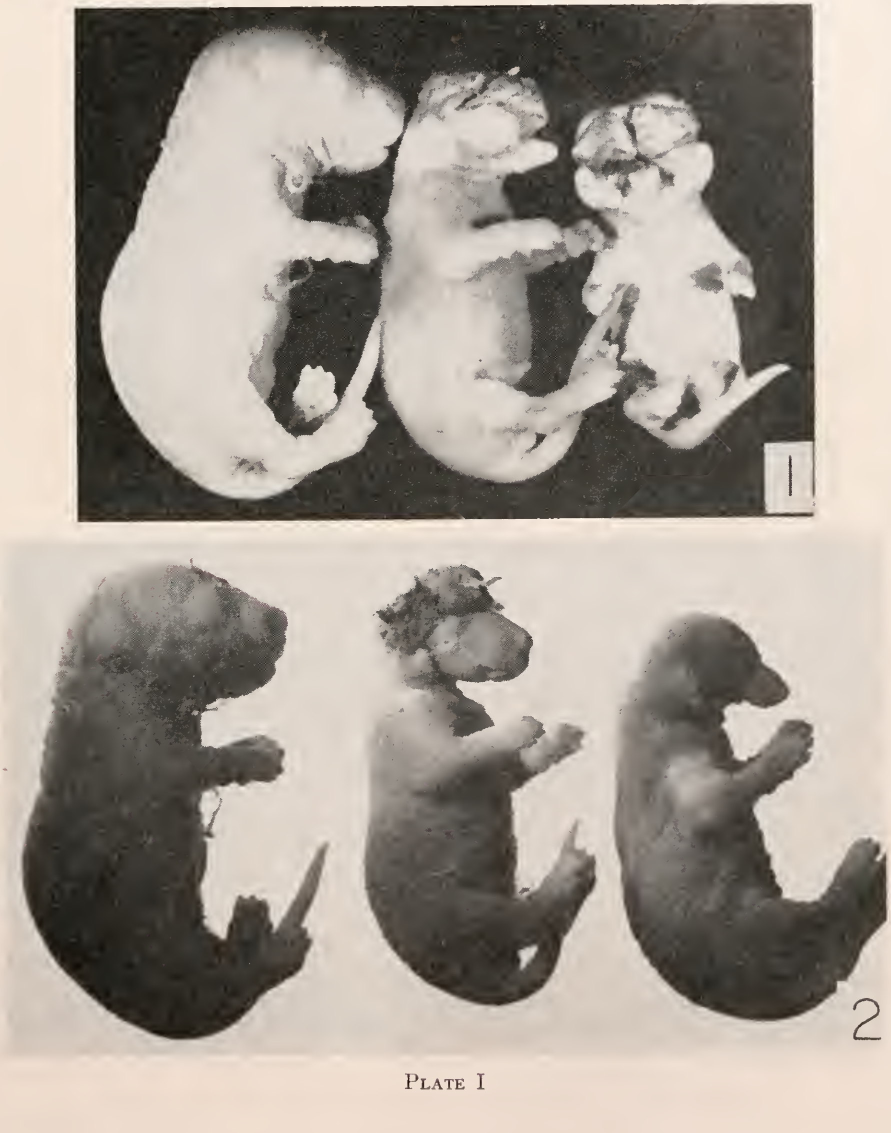
Among the anomalous conditions it is important to point out that the controls are not without congenital anomalies which may well be of genetic origin (Ebert, 1961). However, the control data must be taken as the base line for comparison, in order to determine the additive effect of x-irradiation insult. Such added congenital anomalies are not genetic, but developmental. For instance, resorptions (which generally mean early embryonic death) are about the same in the two control stocks (12% and 13%) but are lower in the controls of all hybrid crosses. This would constitute added evidence of heterosis. When the embryo is x-irradiated at 8.5 days the resorption data range from 18.6% to 92%, indicating great variation in response. The highest level of resorptions occurs among the pure C57 lines (92%), indicating maximum embryonic radiosensitivity, and is lowest among the F_1 generation of the hybrid × hybrid cross. In the previous study (Rugh, Grupp and Wohlfromm, 1961) it was shown to be 90% for the C57 line. When the hybrid generation is crossed to the C57 stock, the resorptions are again high (38%) and lowest when the hybrid is crossed with the hybrid. Thus, as even among the control data, it appears that there is radioresistant heterosis when CF1 and C57 are crossed, and this heterosis is modified by outcrossings in such a way as to indicate that the presence of any CF1 genes is beneficial. When heterozygosity appears to be maximum, the conditions for surviving the radiation insult are the greatest (see Haverland and Gowen, 1960).

TABLE II

Percentage dead and anomaly risk

Group	# Implantations	# Dead & resorbed	# Survivors for CNS anomalies	# & % of CNS anomalies
CF1 × CF1 controls	471	64 (13.6%)	407	0 (0%)
CF1 × CF1 + x-rays	235	105 (44.7%)	130	33 (25.4%)
C57 × C57 controls	253	37 (14.6%)	216	3 (1.3%)
C57 × C57 + x-rays	252	249 (98.8%)	3	0 (0%)
C57/CF1 × C57/CF1 controls	269	20 (7.4%)	249	1 (0.4%)
C57/CF1 × C57/CF1 + x-rays	248	85 (34.3%)	163	7 (4.3%)
C57/CF1 × CF1 controls	273	25 (9.2%)	248	1 (0.4%)
C57/CF1 × CF1 + x-rays	264	95 (36.0%)	169	20 (11.8%)
C57/CF1 × C57 controls	261	21 (8.0%)	240	1 (0.4%)
C57/CF1 × C57 + x-rays	254	127 (50%)	127	2 (1.6%)

Consolidating the deaths, whether late fetal or early embryonic death, with resorption and considering only the survivors as having any *risk* of developing anomalies, it is obvious that the percentage of congenital anomalies of the central nervous system was always greatest when the CF1 collective influence was the greater. The resorption percentages were greatest when the C57 influences were the greater. This sort of experiment does not reveal any specific genetic influences, but rather lumps together the CF1 influences and compares them with the aggregate effect of C57 influences on the development. The anomalies are no doubt developmental, without mutational factors involved, because they arise from x-ir-



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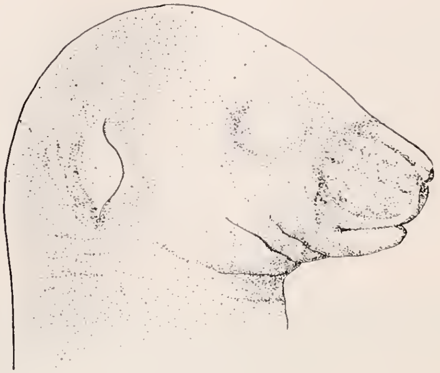

CONTROL

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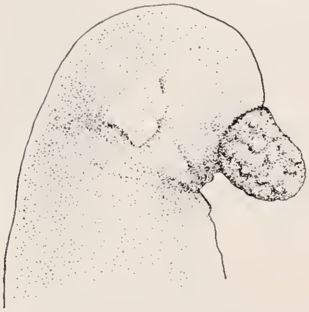
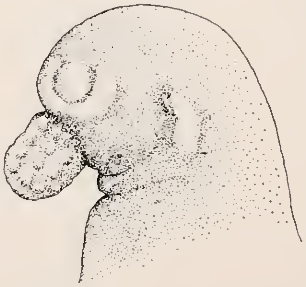


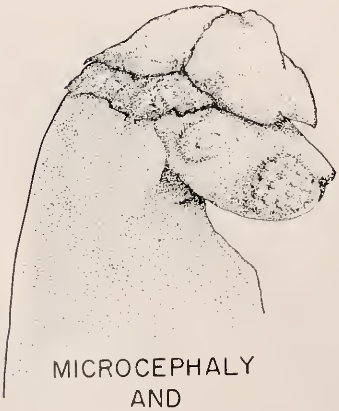
MICROCEPHALY
Right side

4



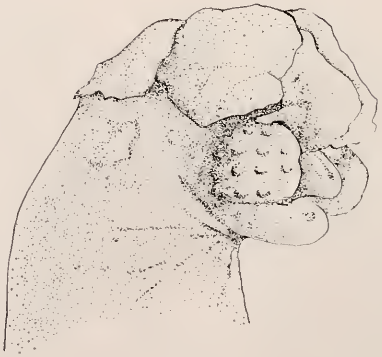
MICROCEPHALY
Left side

5



MICROCEPHALY
AND
EXENCEPHALY

6



GROSS CEPHALIC
ANOMALY

7

X-IRRADIATION-INDUCED
CENTRAL NERVOUS SYSTEM ANOMALIES

PLATE II (drawings by P. Van Dyke)

radiation at almost mid-gestation. However, having indicated a genetic prepotency toward early death and resorption, in the case of the C57 mice, and toward CNS anomalies in the CF1 mice, it is necessary to suggest that this difference may be due to the fact that the basic genotype of the CF1 mice may be hardier (with regard to radiosensitivity) so that when its influences are preponderant, there is a better opportunity for CNS congenital anomalies to develop—simply because there is better survival of those with CF1 genetic factors.

Among the specific anomalies listed, those involving the central nervous system appear to be the most obvious and drastic, probably since 8.5 days represents the time of initial and most active neurogenesis. (See Plates I and II.) However, interesting data appear here in that the incidence of these congenital anomalies is more frequent among the progeny of CF1 mice than among the supposedly more radiosusceptible C57 strain. This is explained in part by the fact that the C57 strain is so radiosensitive that most of them are lost as resorptions. Among those lost were probably many that, had they survived, might have exhibited CNS anomalies. None of the few (1%) surviving x-irradiated C57 mice had gross CNS anomalies. This difference is further borne out by the fact that when the hybrid mice (CF1 × C57) are crossed with the CF1 strain more (7.6%) CNS anomalies develop (because of better survival) than when such hybrids are crossed with the C57 strain (0.8%). All x-irradiations were at 8.5 days' gestation. It is suggested that differences following the back-cross to the two strains may be related to the higher incidence of resorptions whenever the C57 genes are present, thus reducing the fetuses among which CNS anomalies could occur. Other developmental anomalies, such as stunting, anemia, evisceration, and shortened tails, did occur but not in sufficient numbers for evaluation, although the data are suggestive. These conditions cannot be of genetic origin, since the x-irradiations occurred after 8.5 days of embryonic development. They are achieved by x-irradiation interruption of the normal morphogenetic processes, aided by the subsequent deletion of necrotized 'blast cells of the actively differentiating embryo. Since eye anomalies normally occur in pure C57 stocks one would expect the C57 genic influences to be correlated with higher incidence of these defects, but this is not borne out by the data. Again this may be due to the obscuring of this tendency by the presence of CF1 genes which are not predisposed to eye defects. *The question may be asked as to whether it might be better to be so radiosensitive that there is a high death rate rather than to be less radiosensitive and survive, only to develop congenital anomalies.*

Summary and Conclusions

1. Increased radioresistance is manifested in hybrid embryos exposed to 200 r at 8.5 days of embryonic development when divergent stocks of mice are crossbred, as determined by the incidence of congenital anomalies. This is evidence of embryonic heterosis.

2. When hybrid mice are mated with hybrids (of the same crosses) or with either of the parental stocks, it is apparent that the presence of CF1 influence (genes?) affords the embryos more radioresistance only when the cross is with the CF1 line. The presence of CF1 genes brings the results up to the pure CF1 data so that it appears that there is a sort of dominance effect which becomes heterosis when the proportion of CF1 genes exceeds this minimum in these heterozygous embryos.

3. In hybrid combinations the development of CNS congenital anomalies appears to be more frequently related to the presence of CF1 genes than to those from the C57 strain. Conversely, resorptions appear to be more directly related to the presence of the C57 genes. Even in the pure strains this is substantiated because 14% of the CF1 embryos and none of the C57 embryos showed CNS anomalies following 200 r x-rays at 8.5 days' gestation. Likewise, the C57 strain embryos reacted to this exposure by producing 92% resorptions while the CF1 embryos showed only 32%.

4. The data on congenital anomalies seem at first to be confused. This is due to the simple fact of relative radioresistance of the CF1 embryos, which allows them to survive and hence to develop x-irradiation-induced congenital anomalies, particularly of the central nervous system. The radiosensitivity of the C57 strain reduced their survival. As a result of this, there are fewer survivors to develop CNS anomalies.

5. As in other studies in heterosis, it appears that this embryonic hybrid vigor is correlated with maximum heterozygosity and is reduced as this condition is diluted toward either of the parental conditions.

6. Radioresistance (or, conversely, radiosensitivity) of the embryo appears to be closely allied to inherent genetic factors quite different in the two strains of mice. In one strain there is the greater tendency to resorption and death and in the other to survival with attendant development of congenital anomalies.

LITERATURE CITED

Ebert, J. D., 1961. First International Conference in Congenital Malformations. *J. Chron. Dis.*, **13**: 91–132.

Grahn, D., 1958. Acute radiation response of mice from a cross between radiosensitive and radioresistant strains. *Genetics*, **43**: 835–843.

Haverland, L. H., and J. W. Gowen, 1960. Physiological vigor as a factor in radiation reduction of lifetime fertility in mice. *Rad. Res.*, **13**: 356–368.

Nash, D. J., and J. W. Gowen, 1962. Effects of x-irradiation upon postnatal growth in the mouse. *Biol. Bull.*, **122**: 115–136.

Otis, E., 1953. Prenatal mortality rates of seventeen radiation-induced translocations in mice. Univ. Rochester A.E.C. Pub. #291.

Rugh, R., and J. Wolff, 1958. Increased radioresistance through heterosis. *Science*, **127**: 144–145.

Rugh, R., H. Funk and M. Wohlfromm, 1961. Heterosis and testes x-irradiation. *Atompraxis*, **7**: 243–248.

Rugh, R., M. Wohlfromm and E. Grupp, 1961. Evidence of prenatal heterosis relating to x-ray induced congenital anomalies. *Proc. Soc. Exp. Biol. Med.*, **106**: 219–221.

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SUN COMPASS ORIENTATION OF PIGEONS UPON EQUATORIAL AND TRANS-EQUATORIAL DISPLACEMENT

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The operation of the sun compass in northern latitudes has been established in numerous arthropods, reptiles, fish and birds (von Frisch, 1950; Pardi and Papi, 1952; Birukow, 1960, Gould, 1957; Fischer, 1961; Hasler *et al.*, 1959; Braemer, 1959; Kramer, 1952a, 1952b; von St. Paul, 1953). The utilization of this mechanism for actual field orientation has been demonstrated in many arthropods (von Frisch, 1950; Pardi and Papi, 1952 and others) and pigeons (Schmidt-Koenig, 1960, 1961); it has been shown likely that it is used by turtles (Gould, 1957) and fish (Hasler *et al.*, 1960).

A large number of field studies of migratory birds has revealed the prevalent role of directional orientation (Rüppel, 1944; Rowan, 1946; Rüppel and Schüz, 1948; Perdeck, 1958; Lack, 1959, 1960). The actual application of the sun compass during migration has not yet been proven. However, if the sun compass were applied, birds whose migrational routes cover many degrees of latitude or lead to equatorial or trans-equatorial regions would encounter rather striking changes in the sun's angular velocity (both azimuthal and directional). Braemer (1960) particularly has pointed to the complex nature of a full scale sun compass which must operate at grossly different latitudes and different seasons. Something comparable to an almanac would be necessary to master all significant variables.

It is evident that an examination of the performance of the sun compass under extreme solar conditions is inevitable. Southward latitudinal displacement has been carried out in the past with bees by Kalmus (1956) and Lindauer (1959) and with fish by Hasler and Schwassmann (1960).

In this, the first of a series of experiments, the present author examined the operation of the sun compass in a stationary training apparatus in homing pigeons raised at Durham, N. C., upon displacement to Belém, Brazil and Montevideo, Uruguay, in fall, 1961. We are aware of the fact that pigeons as non-migratory birds may well differ in their orientational capacities from migratory birds. This species was, nevertheless, chosen as experimental subject because it handles best in experiments and because the translocation expedition was expected to be too difficult to be accomplished on the first try with small migratory passerines. We will call upon those later.

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Duke University). I would like to particularly acknowledge the special efforts undertaken by both agencies to facilitate the expedition to South America. I am much indebted to Dr. H. O. Schwassmann for his stimulating and helpful role. The experiments reported here were initiated by discussions with H. O. Schwassmann at the 25th Cold Spring Harbor Symposium in 1960. H. O. Schwassmann also must be credited for his effort in the founding of a colony of pigeons at Belém, Brazil, of birds from the Wilhelmshaven and the Duke University strain[1] which will be used for further joint experiments there. I am grateful to Dr. Peter H. Klopfer for his steady helpfulness and support; further, to the late Dr. Walter A. Egler, Director of the Museu E. Goeldi, Belém, Brazil, and his successor Dr. Eduardo Galvão, to Dr. Jose Maria Conduru, director of the Instituto Agronomica do Norte, Belém, Brazil, for their extremely helpful cooperation; to Drs. Paul Ledoux and Werner Sattler (then at Belém) for their kind assistance; last, but not least, to Prof. Dr. Victor Bertullo, director of the Departamento de Investigaciones Pesqueras, Universidad de Montevideo, Montevideo, Uruguay, for his most helpful efforts to facilitate the experiments there. I am grateful to Peter H. Klopfer for critically reading the manuscript.

MATERIAL AND METHODS

In order to avoid the bias of the hand-operated device originally inaugurated by Kramer (1952d), and also used by von St. Paul (1953), Hoffmann (1954; 1960), Rawson (1954), and modified by Rawson (unpubl.) and Schmidt-Koenig (1958; 1960), a semi-automatically operating apparatus for the directional training of pigeons was designed (Fig. 1).

Twelve pecking discs, attached to micro switches, are mounted symmetrically at the periphery of a circular cage, one meter in diameter. Landmarks are screened by a removable aluminum wall. A food cup whose cover can be opened and closed either manually or by a solenoid is inserted into the center of the floor. This portion of the apparatus rests on a base of bakelite upon which it can be rotated.

The base contains 12 electrical contacts, each of which is connected to an electromagnetic counter mounted in a separate box. Also mounted in this box is a 6-volt battery, a relay to operate the solenoid and a time delay to keep the food cup open for several seconds at a time. The contact (*i.e.,* the compass direction) which is to activate the solenoid that opens the cover of the food cup for rewarding a correct choice can be selected by a rotary switch. Each pecking disc is wired to a brush which rests on the contacts in the base. Upon proper rotation of the upper portion (by hand) only that particular disc, the direction of which coincides with the desired training direction, can activate the rewarding mechanism. In addition, each disc activates a counter corresponding to the direction to which it points. The entire apparatus is portable so as to facilitate long-distance transport.

After 3–4 days of starvation, the birds, first, were taught to walk to and peck at a disc and to walk back and look for food in the center cup. This took a pigeon about two hours to learn. Then the bird had to learn to peck at a disc in one particular compass direction (the training direction). Only correct choices activated the rewarding mechanism. This took a pigeon several weeks to learn. During training the cage was irregularly rotated; during the final stages of training, rotation was according to a random number table (the Rand Corporation, 1955) with a range of 1–10 or 5–15 positions. For the training, the apparatus and the birds were transported to various open places in the immediate vicinity of Duke University where the apparatus was set up on top of a pickup truck. At other times the birds were housed in an aviary at the Duke Forest lofts.

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[1] Birds from the Wilhelmshaven strain have been imported to and are being bred at Duke University. Differences in the orientational abilities of the two strains are the subject of another paper.

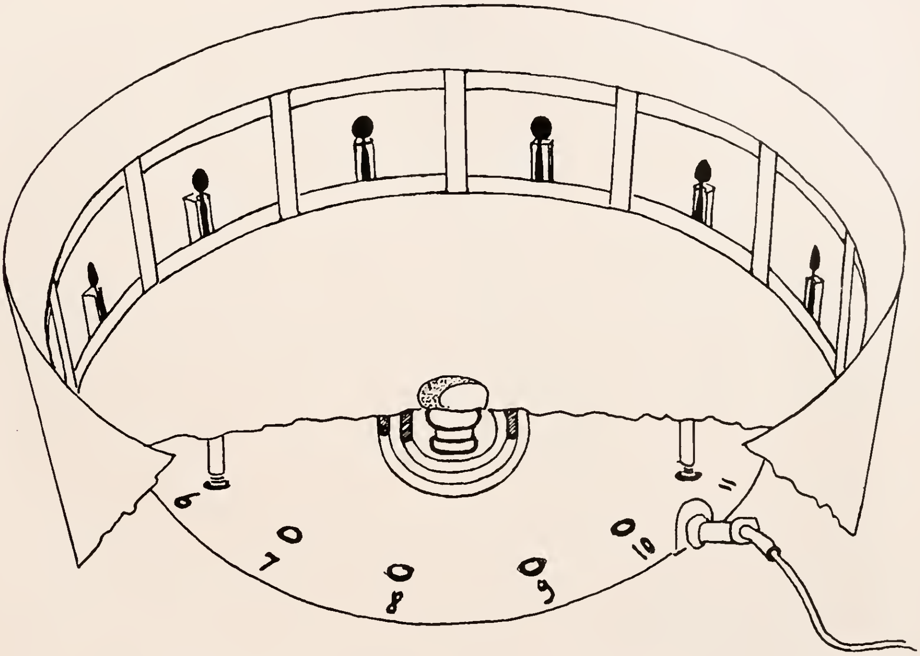

FIGURE 1. Perspective section view of the training apparatus. Six of the twelve black pecking discs are shown above their sockets which house the micro switches. Netting, through which the birds peck at the discs, lines the circular framework around the discs; other netting covers its top. In the center of the upper platform the food cup is indicated. Underneath the upper platform, brushes (hatched) are shown in contact with metal rings centered on the base through which the rewarding is operated. Six of the twelve contacts are depicted in the base (Nos. 6–11) and two of the twelve brushes subtending from the upper platform to contacts Nos. 6 and 11. For more details see text.

Five pigeons, offspring of the Wilhelmshaven strain bred at Duke University, were subjected to directional training on May 2, 1961; the training continued through mid-September, 1961, with 3–4 training sessions per week. The training direction was to the south. The training time varied between 8:45 and 14:45 true local time (Fig. 2). In each session, each bird was allowed to work for about 20 minutes. From June 15, 1961, on, each bird had to perform 5 unrewarded choices, from July 1 on, 10 unrewarded choices, and from August 1 on, 20 unrewarded choices before the rewarded training started. The direction of the mean vector of these unrewarded choices from each session after July 24, 1961, when the training seemed to have taken shape, are given in Figure 2.

In the critical tests in South America the birds had to perform 10–20 unrewarded choices in each session. This took, usually, less than 10 minutes. The cage was rotated irregularly between choices. A little while after the choices, the birds were allowed to feed from the hand-operated cup. There was no training in South America.

The mean vector of all choices of each session has been calculated according to Gumbel, Greenwood and Durand (1953). For statistical evaluation, the procedure and tables of Greenwood and Durand (1955) and Durand and Greenwood (1958) and graph derived from these (Schmidt-Koenig, 1961, appendix), respectively, have been used to discriminate between random and non-random samples. Non-random samples ($p \leqq 0.05$) were plotted in black symbols (Figs. 2–3); those random above the 5% level in open symbols. If statistics could not be applied, due to small sample size ($n < 6$), an open symbol with a central point is plotted. Unfortunately, no specific method to compute the confidence limits of mean vectors is yet available.

Solar and experimental data have been calculated and plotted with reference to true local time (TLT), allowing for the equation of time. All solar data have been taken from the Nautical Almanac for 1961 and the Tables of Computed Altitude and Azimuth (see references).

Experiments and Results

Durham, N. C.

Figure 2 gives the performance of the birds between July 25, and September 14, 1961, at Durham, 36° 00′ N; 78° 56′ W. Three seasonally characteristic sun azimuth curves are also depicted in Figure 2. All birds compensated rather well for the local sun's movement. The majority of choices falls into the range for spring and summer; however, the scatter is too large to decide whether or not the birds are able to allow for the specific seasonal rate of change of azimuth of the sun.

The translocation experiment was planned for the fall equinox of 1961. This would provide the least change in day/night ratio and would permit tests under the zenith sun. Unfortunately, political events in Brazil delayed the travel until the end of September. The birds were trained the last time at Durham on September 14, 1961. From September 25 on, they were accommodated in a covered transportation crate and prevented from directly seeing the sun except during their individual testing periods.

Belém, Brazil

Birds and apparatus were flown to Belém during the night of September 27, 1961. In Belém, the apparatus was set up on top of a flat-roofed building at the Instituto Agronomico (1° 27′ S; 48° 25′ W) which had no tall landmarks around it. A time shift of about two hours clockwise was involved in the translocation. The adjustment of the birds' internal clock is known to take less than two days under natural conditions.

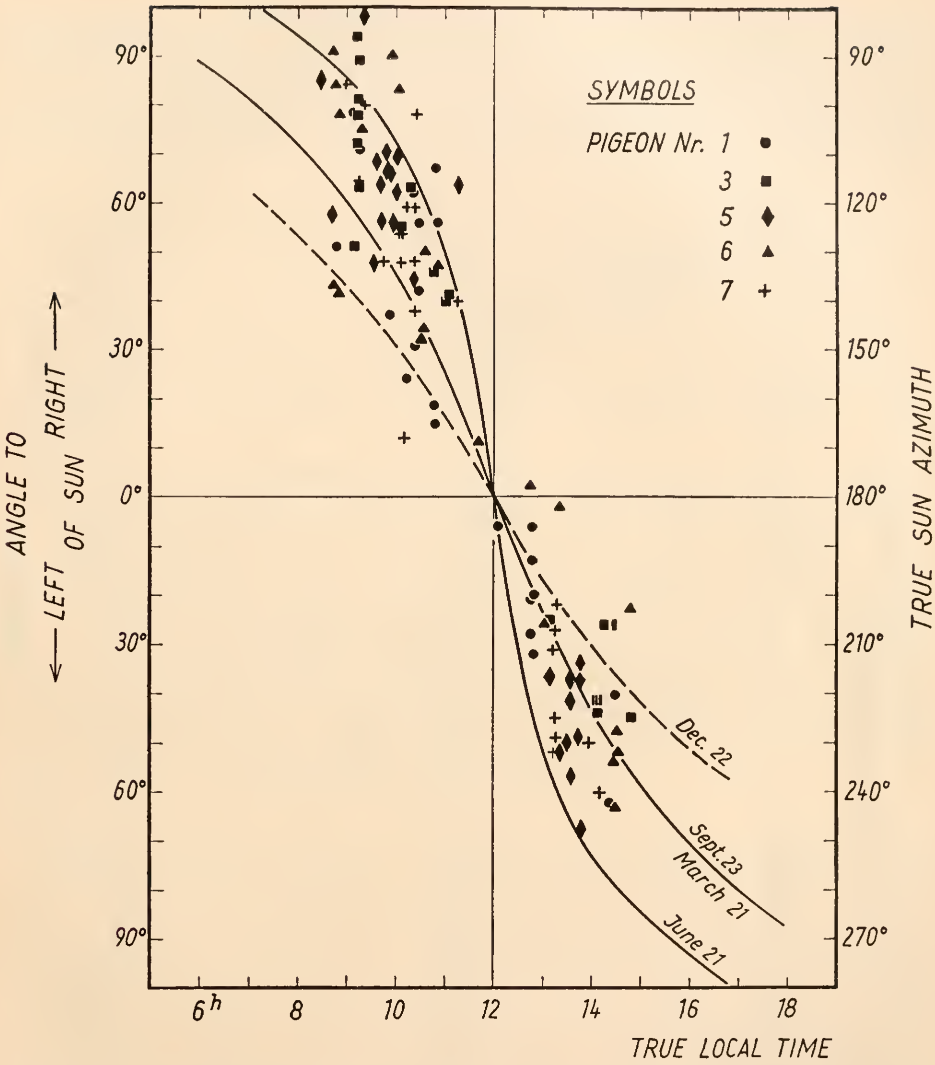

FIGURE 2. The performance of the 5 birds at Durham, N. C., between July 24 and September 14, 1961. Each symbol represents the direction of the mean vector of all unrewarded choices (n = 10–20) during one examination session performed at the time of day indicated on the abscissa and plotted as angle to the actual sun azimuth position (left coordinate). The curves for the summer solstice and for the equinoxes are drawn as solid lines; the birds' training fell in this period of the year.

Birds No. 1, No. 5, and No. 6 were chosen for the equatorial tests. No bird reacted at all in the first testing session on September 29 at 11:50 to 12:22 TLT. From September 30 on, No. 1 and No. 6 cooperated rather well when placed into the apparatus, exceptionally up to three times per day. If the birds were reluctant to peck, this may, at least to some extent and particularly during the first days of examination, have been due to the frequent appearance of vultures in the sky, which obviously frightened and immobilized the birds. No. 5, however, was already very unstable in its readiness to cooperate during its training in Durham. It turned to rather erratic choices in Belém as can be seen from Figure 3. The total number of successful examinations of all birds may be taken from Figure 3.

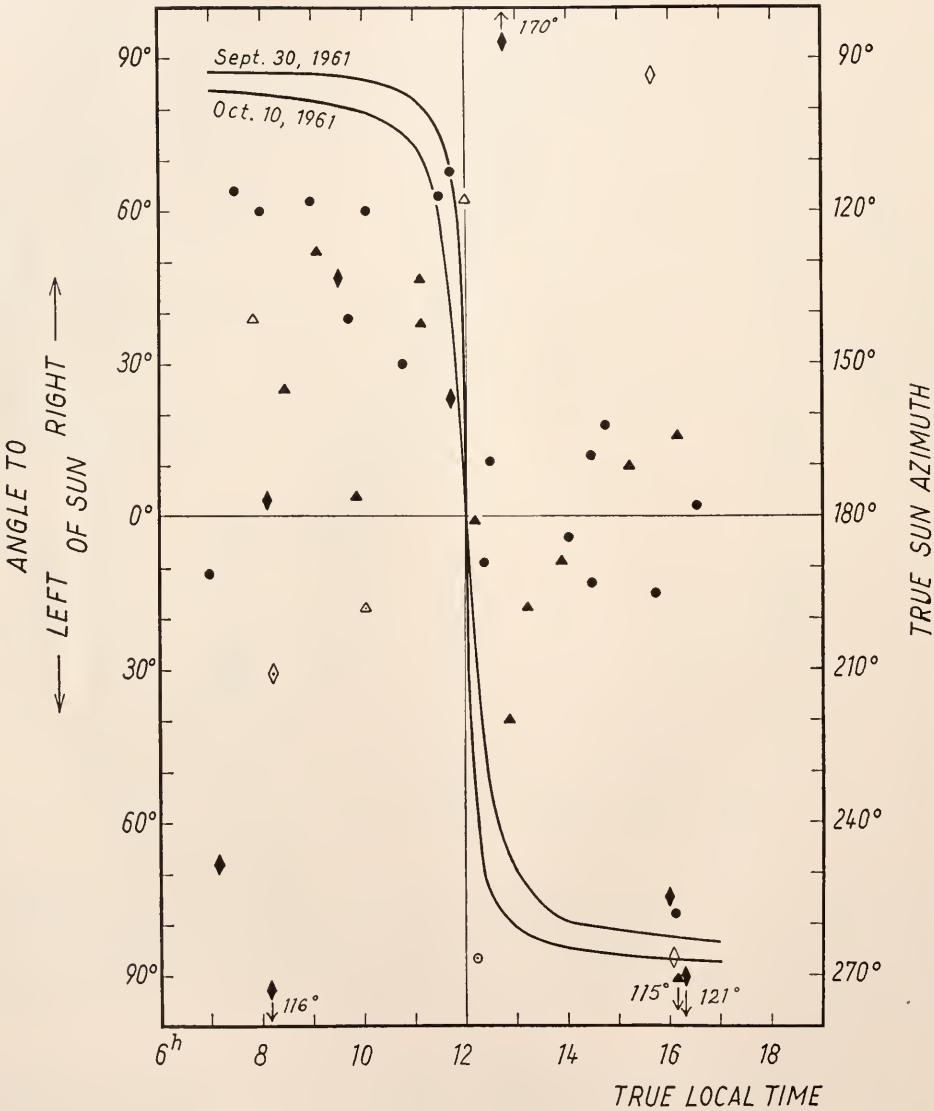

Figure 3. The performance of pigeon No. 1, No. 5, and No. 6 at Belém, Brazil, between September 30 and October 10, 1961. The local sun azimuth curves are drawn for these dates. Symbols and way of plotting as in Figure 2. An open symbol was plotted for random choices.

The last examination was performed on October 10, 1961. Figure 3 also gives the local sun azimuth curves for the first and the last day of examinations.

During the forenoon (neglecting pigeon No. 5), the bird's reference to the azimuth position of the sun conforms with that at Durham rather than with the actual local condition; however, the scatter is very large. The majority of choices performed during the afternoon was well off the usual range. The direction actually indicated by the birds shifted to the west. Hasler and Schwassmann (1960) seem to have encountered a similar phenomenon, although the choices of their fish tended to be at random rather than pointing in particular, however "wrong," directions. It should be indicated at this point that in the Montevideo tests, all birds compensated for the sun's movement much more in accordance with their performance at Durham, also during the afternoon. Pigeon No. 5 worked even more reluctantly than at home. In addition, its few choices were rather erratic. Its scores, therefore, are neglected in view of the more decisive performance of the other birds.

The day of zenith culmination was missed, as pointed out earlier. However, 7 tests of pigeon No. 1 and No. 6 were held when sun altitudes were more than 80°. The respective scores of these tests, which fell within local noon time ± 35 minutes, can easily be located in Figure 3. Only in one instance (pigeon No. 6 at 12:08 at a mean sun altitude of 87°) were the choices ($n = 9$) at random with $p > 0.1$. The other scores that are at random (pigeon No. 1 at 12:16) are due to small sample size ($n = 2$). In the remaining 5 tests at sun altitudes of 81°–84° only two (pigeon No. 6 at 12:18 and pigeon No. 1 at 12:27, both at sun altitudes of 82°) conform with the expectation. Thus, since the whole pattern of scores is rather widely scattered, the precise range of sun altitudes at which the birds are unable to derive compass directions from the sun has yet to be established.

Montevideo, Uruguay

Birds and apparatus were flown from Belém to Montevideo on October 12, and 13, 1961. Tests were performed from October 16 through November 2, 1961, in an open field east of Montevideo at 34° 53′ S; 56° 05′ W. The time shift involved in the translocation was less than one hour counterclockwise. There was ample time for the birds to become synchronized with the local day. Pigeons No. 3 and No. 7 were principally called upon; however, despite their extensive tests at Belém, No. 1 and No. 6 turned out to be still cooperative, as may be seen from Figure 4. Towards the end of the Montevideo time, however, the readiness of all birds to choose was nearly exhausted. The total number of successful tests may be taken from Figure 4. Also plotted in Figure 4 is the mirror image of the sun azimuth curve for Montevideo of October 24, 1961, the medium date of examinations and the azimuth curve for Durham for the same date. The true azimuth curve for Montevideo would fall outside the main figure and is, therefore, plotted in the inserted figure on a full 360° scale.

Again, the birds clearly did not allow for the actual sun movement. The pattern of scores conforms more nearly to that at Durham (Fig. 2); however, the variance appears to be considerably larger. Also, there seems to be a trend to the west around noon and during the afternoon but far less than in the Belém tests. This westward shift may have been caused by the fact that the training at Durham never

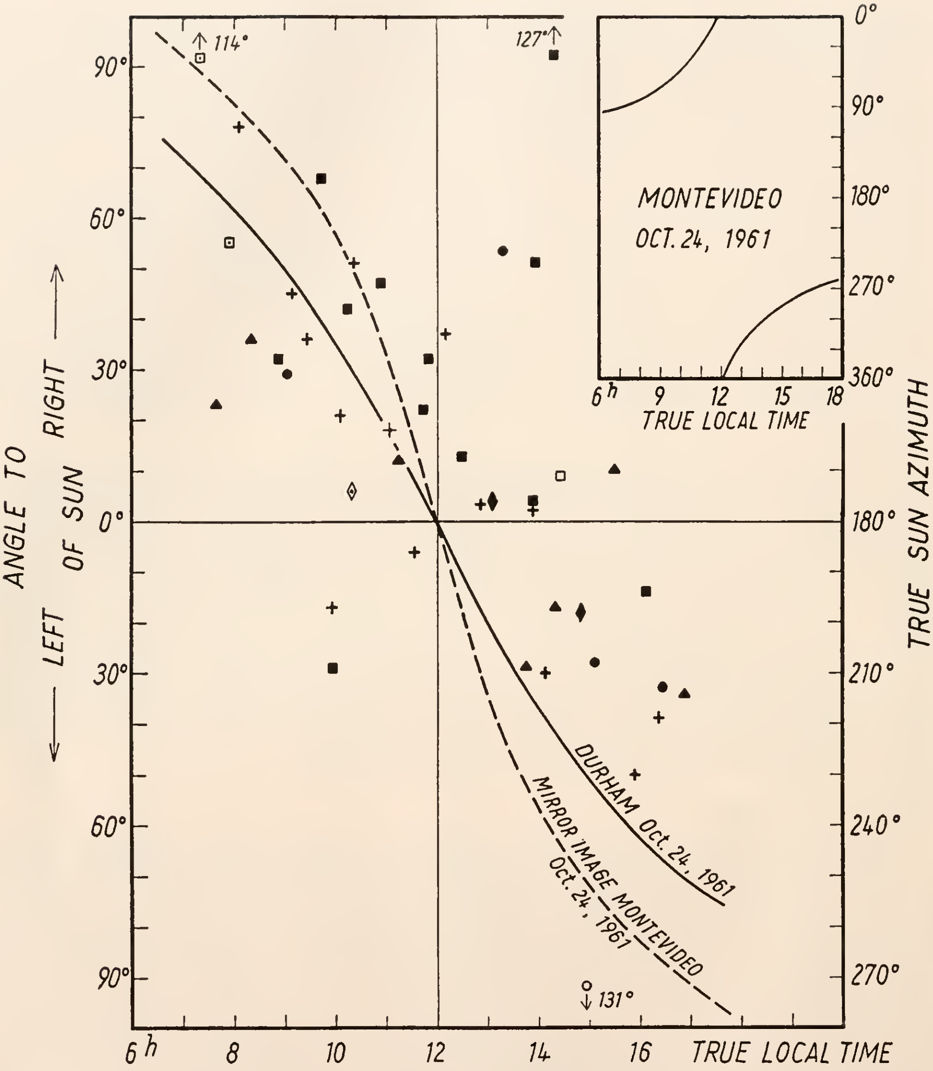

FIGURE 4. The performance of the 5 birds at Montevideo, Uruguay, between October 16 and November 2, 1961. Also plotted in the main portion is the sun azimuth curve for Durham for October 24, 1961 (solid line), as the medium date of examinations and the mirror image of the corresponding curve for Montevideo (broken line). The actual sun curve for Montevideo is depicted on a 360° scale in the inserted figure. Symbols and way of plotting as in Figures 2 and 3.

extended beyond 15:00, while testing at Belém and Montevideo extended until 17:00, involving westerly sun positions. A tendency to follow the sun rather than to maintain a fixed direction and increase the angle to the sun is frequently observed under conditions unfamiliar to the animal.

Discussion and Conclusion

From the scores of the pigeons it can be safely concluded that they did not allow for the equatorial or trans-equatorial sun movement. The birds seem to have largely referred to the sun as if it were the sun at Durham. The scores in South America, though mostly significantly non-random, appear to be more widely spread than those at the training place. It remains unanswered whether this may be interpreted as a specific reaction resulting from the disagreement between orientational features expected by the bird and those actually observed, or as a nonspecific result of handling the bird in unusual and strange conditions, however irrelevant to orientation, or simply as a fading of the training. Nevertheless, the short period of exposure to the local sun of about 10 minutes was clearly insufficient to allow the bird to take full acount of the discrepancies between local solar parameters and those at home. Nonetheless, migratory birds or the navigating pigeons should be able to take account of these within that time period if the sun is the basis for navigation. Previous experiments on the sun compass in northern latitudes failed to demonstrate solar altitude as a source of information for directional choices in starlings and pigeons (Hoffmann, 1954; Schmidt-Koenig, 1958). A number of specifically designed homing experiments did not support solar altitude as providing navigational information to pigeons (Kramer, 1953, 1955, 1957; Rawson and Rawson, 1955; Hoffmann, 1958; Schmidt-Koenig, 1958, 1961).

The homing pigeon, a nonmigratory species, was chosen for this initial series, because of its technical advantages. That it is not an entirely irrelevant subject for studies of sun compass orientation in equatorial regions may best be illustrated by a non-specific account: it is illegal to keep homing pigeons in Brazil because they are extensively used for smuggling diamonds and narcotics. This speaks for their reliability in homing, at the very least.

A new series of experiments is under way in which pigeons, raised and trained at Durham, N. C., will be exposed to the local conditions in South America in an aviary several months in advance of tests. This procedure may reveal whether individuals are able to adjust to grossly different solar conditions. The present series may serve as a control experiment.

In the translocation experiments with bees (Kalmus, 1956; Lindauer, 1959, see also 1960) the examinations concerned offspring of displaced individuals rather than the translocated individuals themselves. Kalmus (1956) claims that even the offspring did not adjust to the new solar conditions. Lindauer (1959) clearly demonstrated in his experiments that the offspring allowed for the equatorial sun movement. This apparent controversy has not been settled yet. Another relevant experiment was concerned with displaced fish (Hasler and Schwassmann, 1960). The authors consider their data as preliminary and exploratory and only drew tentative conclusions. It is unfortunate that the majority of the scores of their fish so far published are in fact not very convincing, due to a sample size just too small for statistical evaluation and to too large variance. This is, in turn, due to specific difficulties arising from work with fish.

Summary

1. Five homing pigeons were directionally trained at Durham, N. C. (36° 00′ N; 78° 56′ W). An automatically recording and rewarding cage was used. The

directional response of the birds was tested upon displacement to Belém, Brazil (1° 27′ S; 48° 25′ W) and subsequently to Montevideo, Uruguay (34° 53′ S; 56° 05′ W). The birds were prevented from directly seeing the sun except for the actual test periods of about 10 minutes on each occasion.

2. The birds clearly did not allow for the respective local sun movements in South America but referred to the sun as if it were the sun at Durham. The choices in South America were more widely scattered than those at home.

LITERATURE CITED

Birukow, G., 1956. Lichtkompassorientierung beim Wasserläufer *Velia currens* F. (Heteroptera) am Tage und zur Nachtzeit. *Zeitschr. Tierpsychol.,* **13**: 463–484.

Braemer, W., 1959. Versuche zu der im Richtungsgehen der Fische enthaltenen Zeitschätzung. *Verh. Dt. Zool. Ges. 1959.* Münster Westf. 276–288.

Braemer, W., 1960. A critical review of the sun-azimuth hypothesis. *Cold Spring Harbor Symposia,* **25**: 413–427.

Durand, D., and J. A. Greenwood, 1958. Modification of the Rayleigh test for uniformity in analysis of two-dimensional orientation data. *J. Geology,* **66**: 229–238.

Fischer, K., 1961. Untersuchungen zur Sonnenkompassorientierung und Laufaktivität von Smaragdeidechsen (*Lacerta viridis* Laur.). *Zeitschr. Tierpsychol.,* **18**: 450–470.

von Frisch, K., 1950. Die Sonne als Kompass im Leben der Bienen. *Experientia,* **6**: 210–221.

Gould, E., 1957. Orientation in box turtles, *Terapene c. carolinensis* Linnaeus. *Biol. Bull.,* **112**: 336–348.

Greenwood, J. A., and D. Durand, 1955. The distribution of length and components of the sum of n random vectors. *Ann. Math. Stat.,* **26**: 233–246.

Gumbel, E. J., J. A. Greenwood and D. Durand, 1953. The circular normal distribution: theory and tables. *J. Amer. Stat. Assoc.,* **48**: 131–152.

Hasler, A. D., R. M. Horrall, W. J. Wisby and W. Braemer, 1959. Sun-orientation and homing in fish. *Limn. and Oceanog.,* **3**: 353–361.

Hasler, A. D., and H. O. Schwassmann, 1960. Sun orientation of fish at different latitudes. *Cold Spring Harbor Symposia,* **25**: 429–441.

Hoffmann, K., 1954. Versuche zu der im Richtungsfinden der Vögel enthaltenen Zeitschätzung. *Zeitschr. Tierpsychol.,* **11**: 453–475.

Hoffmann, K., 1958. Repetition of an experiment on bird orientation. *Nature,* **181**: 1435–1437.

Hoffmann, K., 1960. Experimental manipulation of the orientational clock in birds. *Cold Spring Harbor Symposia,* **25**: 379–387.

Kalmus, H., 1956. Sun navigation of *Apis mellifica* L. in the southern hemisphere. *J. Exp. Biol.,* **33**: 554–565.

Kramer, G., 1952a. Die Sonnenorientierung der Vögel. *Verh. Dt. Zool. Ges. 1952.* Freiburg Brsg. 72–84.

Kramer, G., 1952b. Experiments on bird orientation. *Ibis,* **94**: 265–285.

Kramer, G., 1953. Wird die Sonnenhöhe bei der Heimfindeorientierung verwertet? *J. Ornithol.,* **94**: 201–219.

Kramer, G., 1955. Ein weiterer Versuch, die Orientierung von Brieftauben durch jahreszeitliche Änderung der Sonnenhöhe zu beeinflussen. Gleichzeitig eine Kritik der Theorie des Versuchs. *J. Ornithol.,* **96**: 173–185.

Kramer, G., 1957. Experiments on bird orientation and their interpretation. *Ibis,* **99**: 196–227.

Lack, D., 1959. Migration across the sea. *Ibis,* **101**: 374–399.

Lack, D., 1960. Migration across the North Sea studied by radar. *Ibis,* **102**: 26–57.

Lindauer, M., 1959. Angeborene und erlernte Komponenten in der Sonnenorientierung der Bienen. *Zeitschr. vergl. Physiol.,* **42**: 43–62.

Lindauer, M., 1960. Time-compensated sun orientation in bees. *Cold Spring Harbor Symposia,* **25**: 371–377.

Nautical Almanac for the Year 1961. United States Naval Observatory, Washington, 1959.

Pardi, L., and F. Papi, 1952. Die Sonne als Kompass bei *Talitrus saltator* Montagu (Amphipoda Crustacea). *Naturwiss.,* **39**: 262–263.

Perdeck, A. C., 1958. Two types of orientation in migrating starlings, *Sturnus vulgaris* L. and chaffinches, *Fringilla coelebs* L., as revealed by displacement experiments. *Ardea,* **46**: 1–37.

Rand Corporation, 1955. A million random digits with 100,000 normal deviates. The Free Press, Glencoe, Ill.

Rawson, K. S., 1954. Sun compass orientation and endogenous activity rhythm of the starling (*Sturnus vulgaris* L.). *Zeitschr. Tierpsychol.,* **11**: 446–452.

Rawson, K. S., and A. M. Rawson, 1955. The orientation of homing pigeons in relation to change in sun declination. *J. Ornithol.,* **96**: 168–172.

Rowan, W., 1946. Experiments in bird migration. *Trans. Roy. Soc. Canada,* **40**: 123–135.

Rüppell, W., 1944. Versuche über Heimfinden ziehender Nebelkrähen (*Corvus corone cornix*) während des Wegzuges. *J. Ornithol.,* **92**: 106–132.

Rüppell, W., and E. Schüz, 1948. Ergebnisse der Verfrachtung von Nebelkrähen (*Corvus corone cornix*) während des Wegzuges. *Vogelwarte,* **15**: 30–36.

von St. Paul, U., 1953. Nachweis der Sonnenorientierung bei nächtlich ziehenden Kleinvögeln. *Behaviour,* **6**: 1–7.

Schmidt-Koenig, K., 1958. Experimentelle Einflussnahme auf die 24-Stunden-Periodik bei Brieftauben und deren Auswirkungen unter besonderer Berücksichtigung des Heimfindevermögens. *Zeitschr. Tierpsychol.,* **15**: 301–331.

Schmidt-Koenig, K., 1960. Internal clocks and homing. *Cold Spring Harbor Symposia,* **25**: 389–393.

Schmidt-Koenig, K., 1961. Die Sonne als Kompass im Heim-Orientierunssystem der Brieftaube. *Zeitschr. Tierpsychol.,* **18**: 221–244.

Tables of Computed Altitude and Azimuth I and IV. US Navy Hydrographic Office, Washington 1958.

SODIUM, POTASSIUM AND CHLORIDE IN SELECTED HYDROIDS

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The survival of small, relatively simple animals, in aquatic habitats ranging from fresh waters to marine, poses problems of ion- and water-regulation that still need to be defined in quantitative terms. It has been shown that flatworms, acoelomate "organ-level" forms of wide distribution, appear to have a characteristic ionic content which persists in the face of ionic variations in the environment (Steinbach, 1962a). K content of organisms tends to remain in characteristic amount and high with respect to the environment; Na and Cl are maintained above environmental levels in fresh-water forms, and below environmental levels in marine forms with intermediate conditions in various degrees of dilution of sea water (Steinbach, 1962b).

While the flatworms have achieved an "organ-level" of organization, the coelenterates may be characterized as "metazoa of the tissue grade of construction" (Hyman, 1940). Of the coelenterates, the members of the class Hydrozoa range from the fresh-water hydras, through such brackish-water forms as *Cordylophora* to the many colonial hydroids in marine habitats. Typically marine hydrozoa (Topping and Fuller, 1942) appear to have had little success at colonizing the variable brackish waters of estuaries. On the other hand, *Cordylophora* is widespread in brackish- to fresh-water ponds and *Hydra* occurs commonly throughout the fresh-water habitat.

The growth, general behavior and distribution of *Cordylophora* has been reported extensively (Roch, 1924; Kinne, 1958; Fulton, 1961). Much special information about the hydroids is to be found in the symposium report edited by Lenhoff and Loomis (1961). *Tubularia* has been widely used for studies of regeneration. However, the only basic information about water and salt composition and the mobility of these constituents in Hydrozoa is contained in a paper by Lilly (1955). Lilly demonstrates, in *Hydra*, an influx of Na, K and Br and a permeability to water, and gives figures for Na and K content based on an assumed isotopic equilibrium.

The present studies were undertaken to determine directly the Na and K contents and obtain influx values for *Hydra* in dilutions of Ringer's solution with pond water, and of *Cordylophora* and *Tubularia* in dilutions of sea water.

It is shown that the actual analytical content of *H. littoralis*[2] (the species used

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[2] Both *Hydra littoralis* and *Chlorohydra viridissima* were originally obtained from local supply houses and then cultured in this laboratory. No expert taxonomic identification was made but the specimens certainly fit the descriptions in a general way (*cf.* Hyman, 1931). *Chlorohydra viridissima* is probably very similar if not identical with the European *Hydra viridis* (Hyman, 1929). In general the habitat of *Pelmatohydra oligactis* is very similar to that of *Hydra littoralis.* It may be assumed that *Tubularia crocea* was used although there does seem to be some doubt about the identity of specimens from Woods Hole and from Cape Cod Canal.

here) is at the least double that inferred for *Pelmatohydra* by Lilly, and indicates very slowly exchangeable components of both Na and K.

Insofar as the present studies on *Hydra littoralis* are concerned, it is assumed, with the exceptions noted, that the experimental findings of Lilly on *Pelmatohydra oligactis* apply. On this basis, *Hydra littoralis* is assumed to (1) maintain a constant volume in osmolar concentrations of the external medium ranging from pond water to about 40 millimolar sucrose, (2) possess a marked permeability to water as evidenced by shrinkage in higher concentrations of sucrose, and (3) show an appreciable rate of exchange of Na and K contents with the environmental constituents.

MATERIALS AND METHODS

Hydra littoralis [2] was cultured according to the general procedures outlined by Loomis (*cf.* Lenhoff and Loomis, 1961) in a medium containing Versene, Ca and bicarbonate in Chicago tap water. They were fed *Artemia nauplii* five times a week, except the subcultures set aside for experimental use. In general, *Hydra* were not fed for at least one day prior to analysis. A few observations were made on *Chlorohydra viridissima* cultured in Chicago tap water.

Numerous *Hydra* were shaken loose from their attachments in the culture dish and pipetted with culture medium into previously tared conical, 12-ml. centrifuge tubes. The tubes were then centrifuged 5 minutes at ca. 1600 × gravity and the supernatants decanted. The weight of the "*Hydra* pellet" could then be determined. Carboxyl C^{14} inulin was added in some instances to give some estimate of "non-*Hydra*" space. This "space" presumably includes a portion representing non-drainage of medium from the tube, medium around the animals and possibly medium in the coelenterons of the animals. The specimens of *Hydra* were not crushed by the gentle centrifuging, *Chlorohydra* individuals, especially, acting like rather stiff bristles and the "pellet" showing a correspondingly greater "inulin space" than the pellet of *H. littoralis*. The results are reported as concentrations in the centrifuged pellets, inulin values being indicated to show the magnitude of the corrections that could be made on the assumption that inulin spaces represented space outside the animals.

Cordylophora lacustris, obtained originally from Cape Cod, was cultivated according to the general methods discussed by Fulton (1961), using dilutions of natural sea water from Woods Hole. General growth characteristics of *Cordylophora* in various dilutions of sea water have been reported by others (*cf.* Kinne, 1968); the behavior of the cultures used in these experiments was not markedly different from that described. Growth was very slow in concentrations below 10% sea water and above 50% under the conditions used. Stems with hydranths and with occasional bits of stolons were harvested from the appropriate cultures and immersed in the desired medium, usually identical with that in which they were grown. The specimens could then be placed briefly on hard filter paper, allowed to drain a few seconds and transferred to slips of Parafilm for rapid weighing on torsion balance. This treatment did not appear to disturb the hydranths unduly. Specimens so treated appeared normal five minutes after return to their normal medium. The results reported here for ion contents and ion fluxes refer to the whole stem-hydranth system without attempting to separate the contributions of perisarc, coenosarc, or coelenteron.

Tubularia[3] was obtained from Woods Hole Harbor and from the north end of the Cape Cod Canal. There is no assurance that all were of the same species—the classification appears to be somewhat in doubt. However, no differences were noted between animals from the two sources and hence no precise identification was attempted.

In general, *Tubularia* was treated and prepared for analysis according to the methods outlined for *Cordylophora*. *Tubularia* hydranths are very large, plentifully endowed with gonophores during the season of these investigations and inclined to drop off the stems after a day or so at sea-water-table temperatures (19–21° C. during the summer of 1962). Sections of stems, however, seemed to remain in good shape, starting to regenerate new hydranths after loss of the original ones. Therefore, the results reported herewith relate only to stems, rather than to whole stem-hydranth units.

Analysis of ionic contents and measurement of ion fluxes involved the routine methods reported in other studies (*cf.* Steinbach, 1962a).

Table I

Ionic composition of Chlorohydra viridissima *and* Hydra littoralis. *Concentrations in millimoles/kg. Corrected concentrations were calculated assuming inulin space is not part of the animal. The inulin space in parentheses was from another experiment on the same batch of animals. Medium in all cases <1.0 mM in salt.*

	Pellet			Inulin Space %	Corrected		
	Na	Cl	K		Na	Cl	K
Chlorohydra	9.3		11.0	47	17		21
	5.1	1.5	10.3	66	15	4	30
Hydra	9.5	3.4	24.0	37	15	5	38
	18.0	5.7	35.0	(30)	26	8	50

Na and K were assayed by flame photometer on dilute hot acid extracts of the animals. Cl was determined by a Cotlove-type chloridemeter, and Na^{24} and K^{42}, used to study ion fluxes, were assayed with a thin window or NaI well counter, corrections for radioactive decay being made where necessary.

Results

A. *Hydra*

Table I gives the analytical figures for two determinations on *Chlorohydra viridissima* and two on *H. littoralis* made during the same season. While *Chlorohydra* pellets have lower ionic concentrations than *H. littoralis*, "correction" for inulin spaces brings the values closer together. In view of the complexity of *Chlorohydra*, with its symbiotic algae, the rest of the results reported will concern *H. littoralis*.

Although no tests for acclimation to increased salinity over long times were attempted, *H. littoralis* can withstand concentrations up to 20% of amphibian

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[3] See footnote 2.

TABLE II

Ionic contents of pellets of H. littoralis *following exposure of the animals for 30 minutes or more to diluted frog Ringer's solution. Concentrations in millimoles per liter, or per kilogram with the number of analyses averaged in parentheses. Average inulin space in comparable experiments = 31%.*

Solution			Pellet		
Na	Cl	K	Na	Cl	K
0.2	—	<0.1	14 (3)	6 (3)	32 (3)
6.0	7.2	<0.1	14 (5)	10 (5)	35 (5)
9.0	9.6	2.0	18 (5)	12 (5)	26 (5)
20.0	22.0	2.5	24 (4)	17 (4)	38 (4)

Ringer's solution. Loomis (1959) reports normal growth in 5% sea water. Table II gives the analytical results on *Hydra* held half an hour or more in solutions of the composition indicated. There is a remarkable constancy of K concentrations, regardless of either total ionic strength or K content of the bathing medium. Na and Cl contents both increase with increasing external concentration

TABLE III

Entry of Na^{34} *into* Hydra littoralis. *Influx expressed as counts/minute/kg. pellet. Na concentrations: mM/kg. pellet or medium. Per cent exchange calculated as specific activity of pellet ÷ specific activity of medium × 100. Medium: dilute frog Ringer's.*

Immersion time	Na^{24}	Na (total)	Per cent exchange
1.00 hour	3.9	35	37
3.15 hours	3.7	23	53
18.50 hours	4.1	19	73
44.00 hours	4.3	18	80
Medium	6.0	20	100

TABLE IV

K^{43} *influx. Pellet of* H. littoralis. *Solutions of composition as indicated in millimols/liter. Figures as in Table III.*

	K^{42}	K	Per cent exchange
Solution	297	0.9	100
Na = 1, K = 0.9			
6 hours	1630	26	19
24 hours	4230	31	42
Na = 9, K = 0.9			
6 hours	1730	37	14
24 hours	4450	43	31
24 hours	5050	45	34

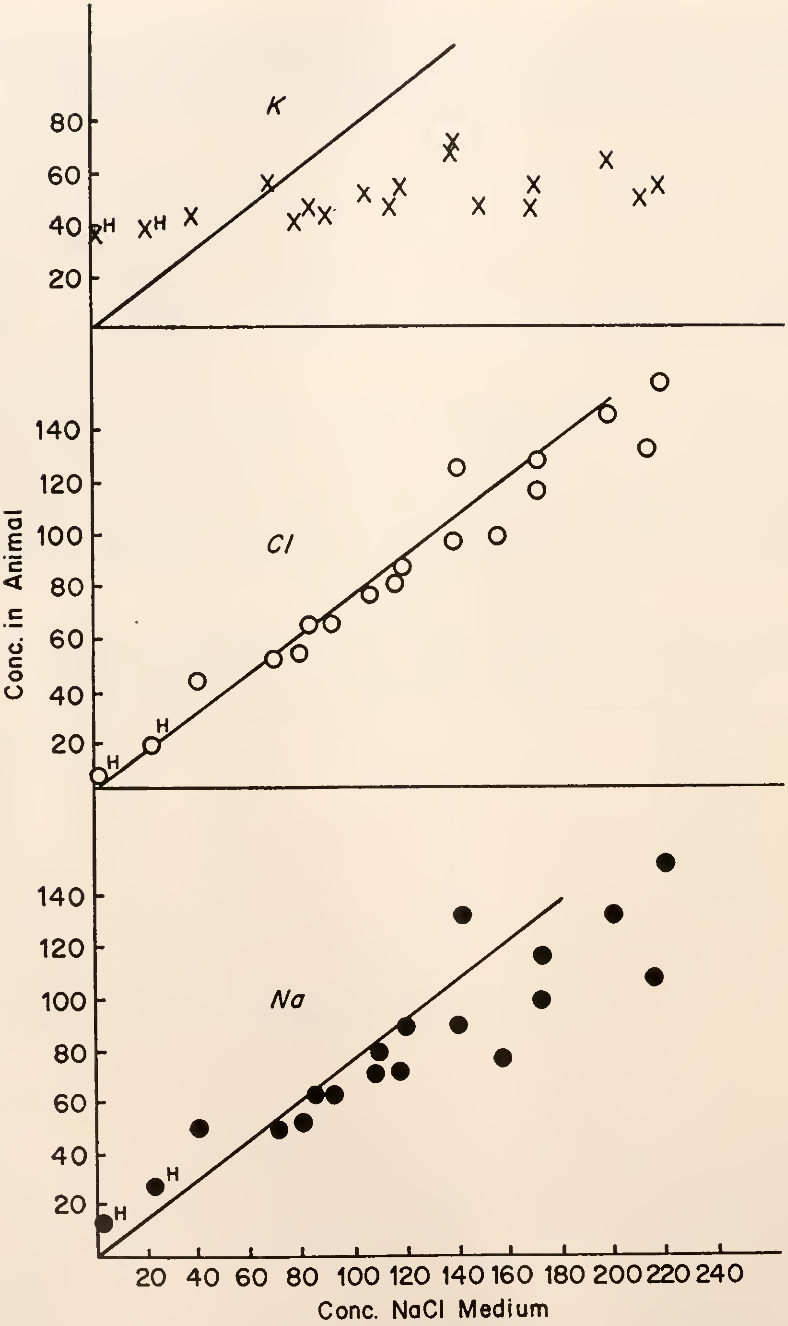

FIGURE 1. Concentrations (mM/kg.) of Na, Cl and K in *Cordylophora*, determined as described in the text, plotted against ionic strength of the medium expressed as concentration of NaCl (mM/L). Points represent single determinations except those marked "H" = values for *Hydra* from Table II. Straight lines have a slope of 0.75.

but in a manner indicating only partial distribution in the pellet. Presumably the volume of the animals did not change appreciably upon transfer to solutions in the osmotic range used (see Lilly, 1955).

Na^{24} and K^{42} both penetrate *H. littoralis*, but slowly (Tables III and IV). Results to date are not precise enough to give reliable rate figures but the half-times for exchange with Na and K of the whole pellets are in the range of 15 to 30 hours, the longer time being indicated for K. The tables also give evidence of a rapid early entrance of Na^{24}, which may be due to entry of Na^{24} into the coelenteron or other extracellular areas. In one experiment on penetration of Na^{24} from a very dilute pond-water medium, there was some evidence of a nearly completely unexchangeable fraction of Na.

B. *Cordylophora*

Cordylophora specimens allowed to grow in various dilutions of sea water were analyzed. The results are summarized in Figure 1. Na and Cl content vary directly with the environment, the general trend indicating a possible equilibration with 50–60% of the volume of the animals. K, on the other hand, remains remarkably constant in concentration, regardless of the external ionic strength. Whether a constant concentration of K also means a constant amount per cell it is not possible to say, since relative weights of the animals in different media were not determined. As the sea water dilutions were made with distilled water, the K concentration of the environment also varied from a high of about 5 millimolar to

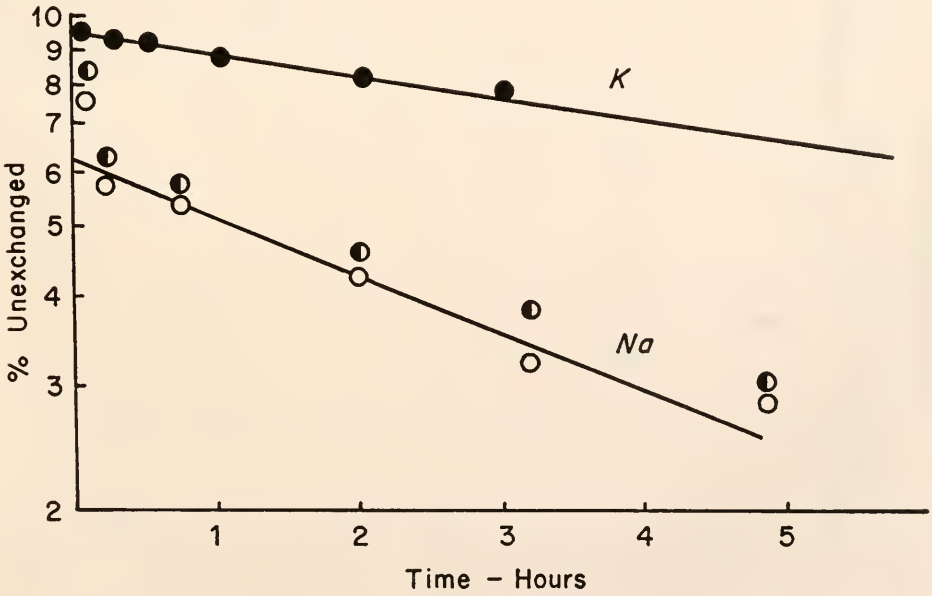

FIGURE 2. Semi-log plot of exchange of Na^{24} and K^{42}, medium to *Cordylophora* against time. Open circles, NaCl concentration of medium = 140 m*M*, half-filled circles, NaCl concentration = 80 m*M*. Dots = K influx from medium 4 m*M* K and 140 m*M* Na. Ordinates = % × 0.1.

TABLE V

Na, Cl and K concentrations (mM/kg.) of Tubularia *stems immersed in solutions indicated for 4 or more hours. Dilutions of sea water with distilled water. All concentrations expressed as averages of 5 determinations with standard errors of mean indicated.*

Medium	Na	Cl	K
Sea water	283 ± 21	345 ± 23	71 ± 1
75% sea water	206 ± 12	253 ± 11	54 ± 2
50% sea water	105 ± 14	165 ± 7	47 ± 1

less than one millimolar. Therefore, the internal K concentration reflects neither a constant inside/outside ratio nor a variation representing osmotic adjustment.

Influx of Na^{24} or K^{42} was measured by removing a standard sample of *Cordylophora* stems at intervals after addition of the appropriate isotope to the medium, blotting gently on hard filter paper and counting directly under a thin-window counter. The animals survived this handling well, most of the hydranths appearing intact after a half dozen or more measurements during the three- to five-hour period of the experiment. At the end of the period indicated, the selected samples, and other samples not subjected to the repeated handling, were extracted, and the extracts analyzed for the ionic constituents and radioactivity as usual. On the basis of the specific activities so determined, the points plotted in Figure 2 were determined.

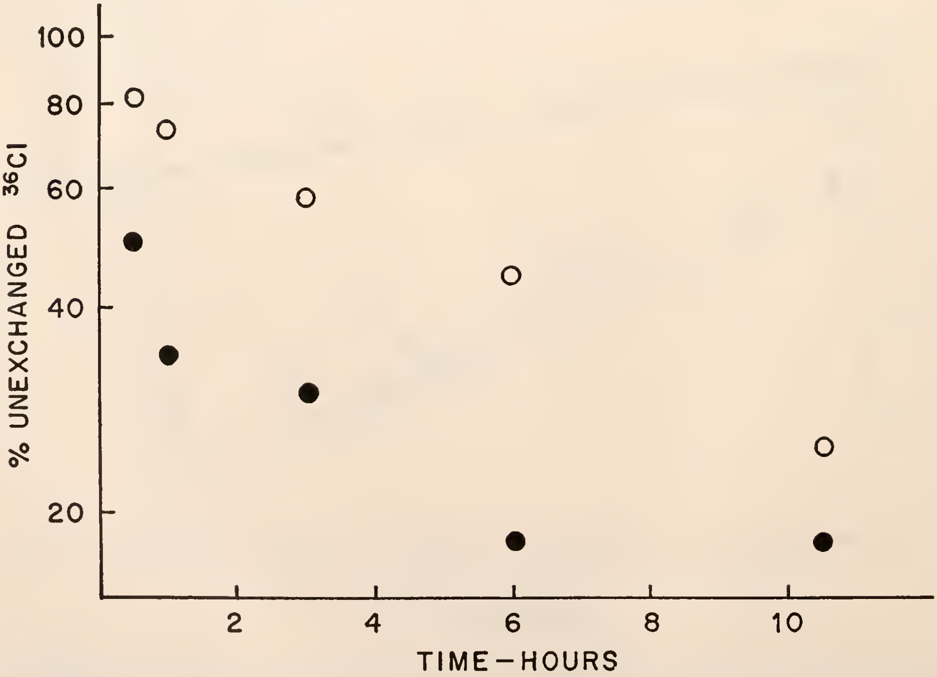

FIGURE 3. Influx of Cl^{36} into *Tubularia* stems. Circles: influx from propionate lobster Ringer's (Cl = 77 m*M*). Dots: influx from normal sea water (Cl = 520 m*M*).

K^{42} exchanges slowly from a medium containing 5 millimolar K and 140 millimolar Na. An estimated minimum half-time for exchange would be of the order of eight hours. During the three-hour period indicated, influx appears to follow a simple kinetic curve, disregarding a very small initial rapid uptake. The time period was not sufficient to indicate whether or not there was an apparently unexchangeable fraction of K. Na^{24} exchanges more rapidly (half-time *ca.* 4 hours for the slow phase) and shows a very rapid initial influx. There is some indication that there is a portion, about 25%, of the total Na that is very slowly exchangeable. Influx of Na is at nearly the same rate from 80 millimolar and 140 millimolar external Na concentrations.

C. *Tubularia*

Because of the large size and relative delicacy of the hydranths of *Tubularia,* the results reported will relate only to analyses of stem segments. The data in general, however, apply to hydranth material, the major differences being those that can be accounted for by the smaller inulin or sulphate space for hydranths (17%) as compared to stems (28%). The perisarc of the stems may be regarded as essentially inert with respect to passage of materials. A few experiments in which empty perisarc tubes were loaded with K^{42} sea water showed nearly complete equilibration with an outside medium in five minutes. Insofar as possible with the available material, straight clean stem segments were used. However, some of the colonies, especially later in the season, had extensive branching patterns.

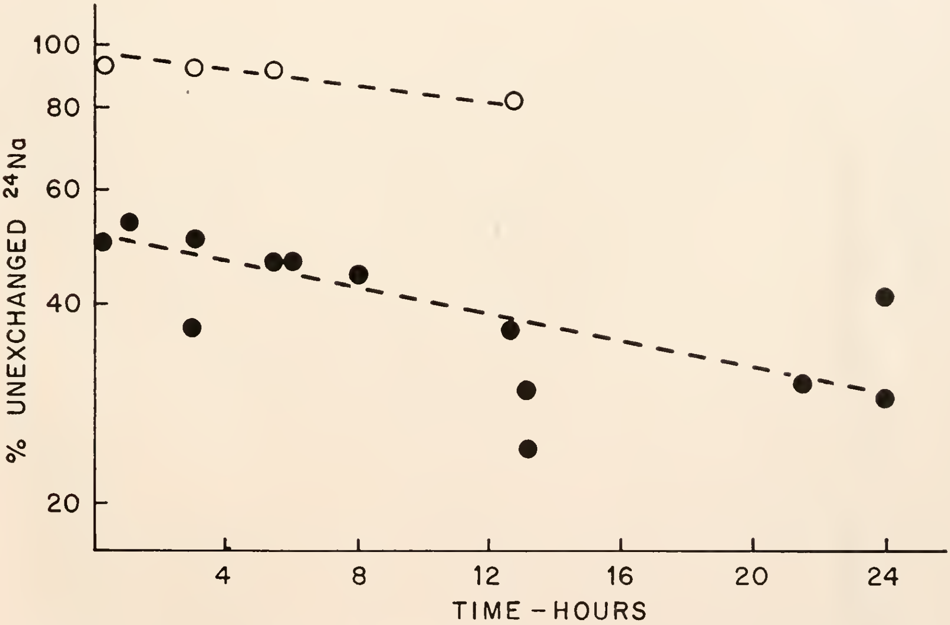

FIGURE 4. Influx of Na^{24} into *Tubularia* stems. Circles: from choline chloride artificial sea water (MBL formula), Na = 25 m*M*. Dots: from normal sea water, Na = 440 m*M*.

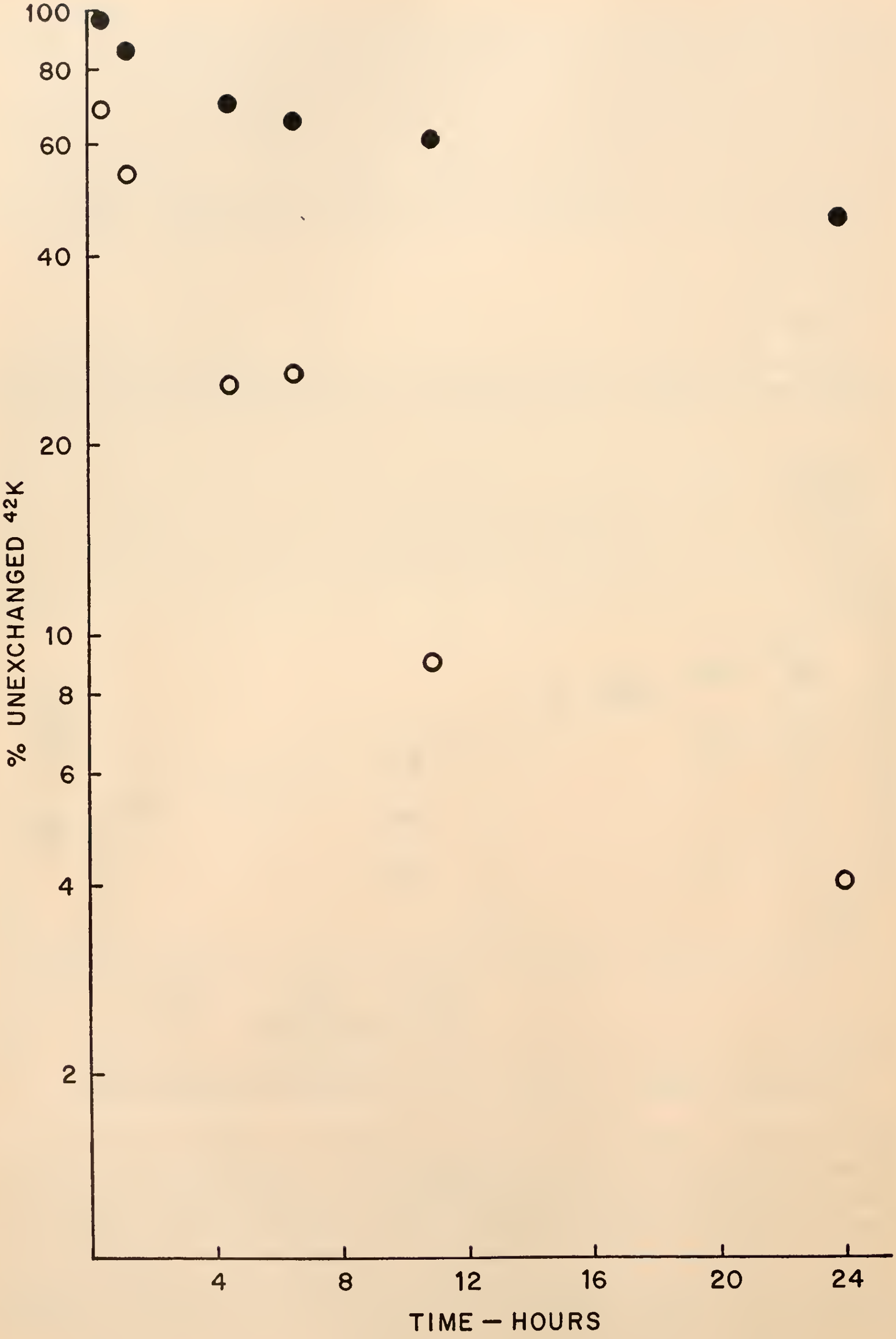

FIGURE 5. K^{42} influx into *Tubularia* from normal sea water (dots) and sea water made 60 m*M* with added KCl (circles).

Table V gives average values for contents of *Tubularia* stems maintained in normal sea water, and in 75% and 50% dilutions of sea water with distilled water. A few measurements indicated only slight swelling of the tissues; thus, the ionic changes probably represent movement of the respective elements rather than dilution effects due to water intake.

Na and Cl concentrations of *Tubularia* stems are quite variable, as indicated by the standard errors of the means. K concentration is markedly more constant in the

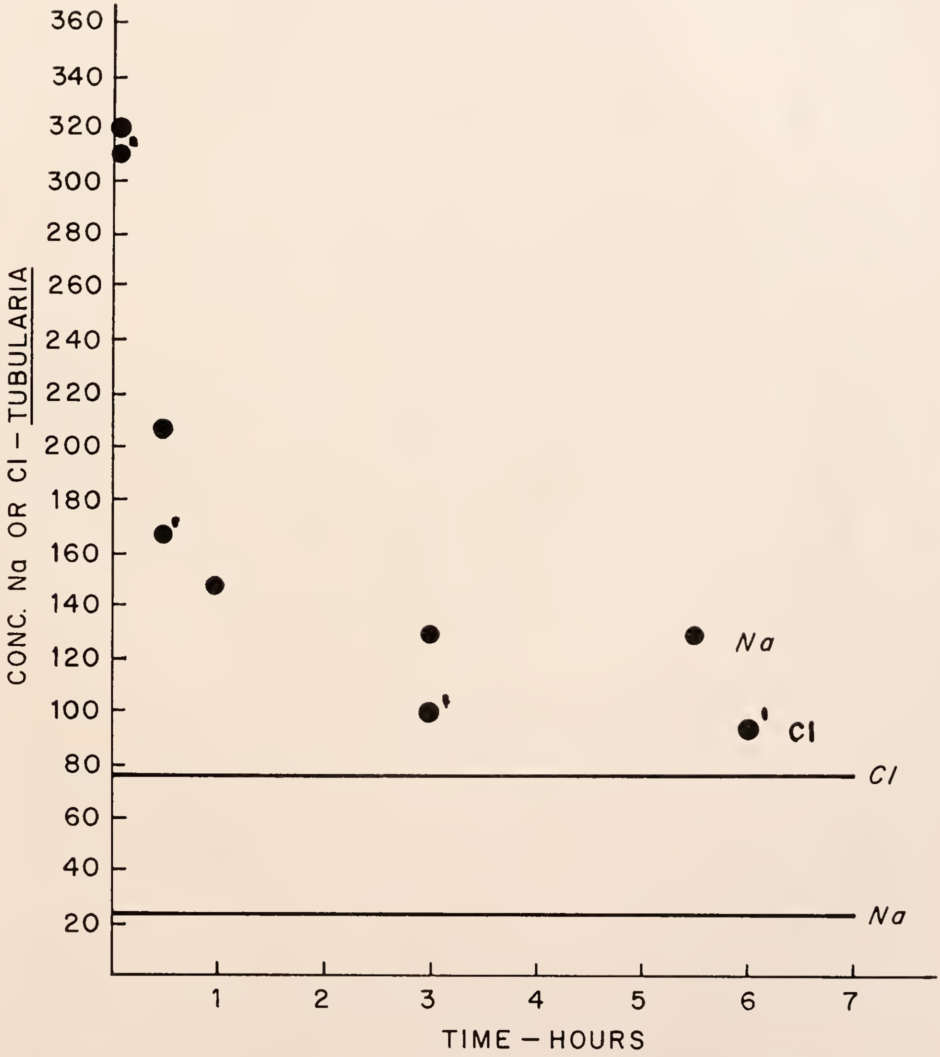

FIGURE 6. Concentration of Na or Cl (m*M*/kg.) in *Tubularia* stems transferred at zero time to Cl-poor medium (propionate lobster Ringer's) or Na-poor medium (choline chloride sea water). Horizontal labelled lines indicate concentrations of respective ions in medium. Time: hours immersion in experimental fluids.

TABLE VI

Concentrations of Na, Cl and K and % exchange of Na^{24} in Tubularia *stems held at sea water table temperature* (ca. *20° C.*) *for the times indicated. Concentrations and exchange values expressed as in Table III. Alcohol-treated stems removed at 24 hours, immersed in 95% ethanol 30 seconds and then returned 2 hours to Na^{24} sea water.*

Time	Na conc.	% Na^{24} exchange	Cl conc.	K conc.
13 hours	258	76	332	85
24 hours	194	87	276	62
32 hours	213	93	291	81
48 hours	193	103	280	81
Alcohol-treated	368	100	444	<10

different batches of stems analyzed. In a few cases, $Na_2S^{35}O_4$ or C^{14} inulin was added to the equilibration medium. As with the other hydroids, assay for S^{35} or C^{14} after an hour or so equilibration indicated a "space" of about 30%.

Na, K and Cl are all exchangeable, at least in part, as indicated by influx of the appropriate isotopes into *Tubularia* stems (Figs. 3, 4, 5).

Cl^{26} penetrates rapidly at first from normal sea water, the initial phase involving more of the total Cl than can be accounted for on the basis of 30% extracellular space (Fig. 3). Testing influx from lobster Ringer's fluid made with sodium propionate[4] instead of the normal NaCl (Cl = 77 m*M* rather than 520 m*M*) has the major effect of decreasing the initial rapid phase, the slower delayed phase of penetration ($t_{\frac{1}{2}}$ = 5–6 hours) being about normal. There is, of course, a net loss of Cl (Fig. 6) during the equilibration period, the major change again occurring rapidly. Cl of *Tubularia* appears to exist mainly in freely diffusible form but involving at least two "compartments" which do not appear to be equated to sulphate and non-sulphate space.

TABLE VII

Effect of excess KCl on exchange of ions; medium to Tubularia *stems. Exchange calculated as in Table III with appropriate isotope measured. K-sea water = 5% M/l. KCl. Time: 6–7 hours. Single determinations of paired runs for each isotope.*

	% Exchange		
	Na^{24}	Cl^{36}	K^{42}
Normal sea water	75	80	35
K-sea water	65	76	74

Na^{24} penetrates from normal sea water (Fig. 4) with a rapid initial phase, mostly accounted for by penetration into sulphate space, followed by a very slow phase ($t_{\frac{1}{2}}$ = > 15 hours). Long-term experiments showed complete equilibration at the end of 48 hours (Table VI); stems immersed 30 seconds in absolute ethanol and then returned to the radlioactive medium took less than two hours (shortest time measured) for complete equilibration.

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[4] I am indebted to Dr. Harry Grundfest for a supply of this medium.

Penetration of Na^{24} from an artificial sea water made with choline chloride instead of NaCl (Na = 25 m*M* instead of 440 m*M*) shows a loss of the first rapid phase of penetration and a slower phase ($t_{\frac{1}{2}} = < 40$ hours). Immersion of *Tubularia* stems in choline sea water leads to an initial rapid net loss of Na (Fig. 6), the tissue concentration at the end of 6 hours still being notably above that of the medium.

K^{42} penetration from normal sea water appears to be relatively uncomplicated. The experiments do indicate a fast, followed by a slow influx into the tissues. Penetration of K^{42} from sea water with excess KCl (K = 60 m*M* instead of 10 m*M*) indicates the drastic speeding of the slow phase. This effect of excess K of the medium is specific for influx of K. Excess K of the medium has little effect on the influx of Na or Cl (Table VII).

Raising the K concentration of the medium increases that of the tissue slightly to a new level which is then maintained (Table VIII). Assuming a 30% extracellular space, cell concentration is raised only from 97 m*M* to 105, the K_i/K_o ratio changing from approximately 10 to 1.7.

TABLE VIII

Effect of immersion of Tubularia *stems in normal 95% sea water and 95% sea water plus 5% by volume M/l. KCl. Figures in parentheses indicate number of separate determinations in averages.*

Immersion time	Na	Cl	K
Normal Sea Water			
<1 hour	276 (2)	331 (2)	72 (2)
4–7 hours	258 (4)	320 (4)	71 (4)
60 m*M* KCl Sea Water			
<1 hour	255 (2)	330 (2)	90 (2)
4–7 hours	245 (4)	328 (4)	92 (4)

DISCUSSION

A striking fact emerging from the present studies is the relative constancy of K concentration in the hydroids investigated, ranging from the fresh-water *Hydra littoralis* in pond water and diluted frog Ringer's, through the brackish-water *Cordylophora lacustris* in various dilutions of sea water, to the strictly marine *Tubularia*. The results, to be sure, represent analyses of whole organisms with contributions of extracellular materials (perisarc, mesogloea and cavities) and undrained fluid of pellets or stems. However, C^{14} inulin studies indicate a free diffusion space of the order of 30% for all types of animals for short immersions, and the probability is that correction for extracellular space, while increasing the concentration values, would not destroy the uniformity.

The only other report known to me on K concentration of the hydroid phase of hydrozoans is that of Lilly (1955) who inferred concentrations in *Pelmatohydra* from influx curves for Na^{24} and K^{42}. While it is possible that the low values she reported represent species differences, it seems probable that her assumption of complete equilibration with external K^{42} is responsible. Whether the very slow rate of equilibration of intracellular K with externally applied isotope indicates any real "binding" cannot be proven from the data. The flux data are reminiscent of the situation found for K influx into whole frog muscle (*cf.* Harris, 1957).

Further studies are needed to determine water fluxes. However, assuming that the high permeability to water deduced from osmotic experiments on *Hydra* (Lilly, 1955) is a condition common to the hydroids, then their osmotic and ionic problems would appear to be as follows:

1. In the range, pond water to 40 millimolar sucrose (*ca.* 5% sea water), *Hydra* volume remains constant. Growth rates are similar in pond water and 5% sea water (Loomis, 1959). The environmental osmotic range for constant body volume may be much broader for *Cordylophora*.
2. There is a remarkable relative constancy of K concentration of the animals in all external salt concentrations tested. *Hydra* living in pond water (NaCl equivalent <0.1 m*M*) has over half the K concentration of *Tubularia* from sea water (NaCl equivalent >500 m*M*). This indicates a K uptake mechanism and a K-regulating mechanism not primarily controlled by internal/external ratios nor by total ionic strength of the medium.
3. With *Hydra*, and *Cordylophora* in very dilute sea water, Na and Cl are slightly in excess of the environmental concentrations. At higher environmental salt concentrations, Na and Cl appear to penetrate freely into about 60% of the volume of the animal. The findings with *Hydra* indicate an Na uptake mechanism in dilute solutions. There may be an Na extrusion mechanism in concentrated salt media. If, however, there is an ion extrusion mechanism, it is a rather odd one, behaving as though it could deplete about half the cell volume of the animal of Na and Cl regardless of the external concentration. The situation has points of similarity to that described for *Tetrahymena* (Dunham and Child, 1961).

The behavior of K seems clear and unequivocal. There is a K concentration (and probably an amount of K per unit cell type) that is fixed and is maintained regardless of external ionic strength or without relevance to a particular K_i/K_o ratio.

It has been suggested that, widespread throughout the animal kingdom, there is an optimal internal K concentration on the order of 150 millimolar (Steinbach, 1962b). Fresh-water invertebrates have long been known to be more dilute forms with respect to tissue K concentration. While careful correction of the whole-animal K concentrations found in the hydroids for "extra-cellular" space would give higher values for cellular K than for whole-animal K, it is doubtful that they would more than approach 100 millimolar. It will be of interest to see whether there is a characteristic "minimal level" of K concentration necessary for the continued existence of animal cells living even in the most dilute media.

There is as yet no conclusive evidence of any extensive "binding" (= immobilization) of ions of the alkali metals in protoplasm. Analysis of flux rates in a variety of forms has indicated some heterogeneity of both cellular Na and cellular K but on the basis of the data currently available, it must be assumed that the cellular Na and K of any living system are diffusible and constitute a major portion of the osmotic concentration of the cells. It thus seems likely that the internal osmotic pressure of *Hydra* cells is even greater than the apparent isotonic point of 40 millimolar sucrose indicated by the shrinkage of the animals in higher concentrations (Lilly, 1955). On the basis of the chemical analyses reported here, the true osmolar concentration would be nearly double the figure cited.

Without in any way intending to suggest that cellular mechanisms for ionic uptake and regulation of internal contents are understood, the water balance of the fresh-water forms presents the most challenging picture. With *Hydra*, for example, water balance might conceivably be achieved by a very impermeable outer layer with a filtration-resorption system in the endoderm-lined gastrovascular cavity. This seems unlikely, both in view of Lilly's studies on tentacles of *Pelmatohydra* and in view of the fact that the *Hydra* can survive yawning periods of up to half an hour with, presumably, free diffusion access of the gastrovascular cavity to the very dilute environment. This hydroid system deserves careful study as a possible example of a tissue system showing water transport without excretory systems on the organ level nor constant cellular organelles such as contractile vacuoles. On the other hand, hydroid tissues are richly endowed with vacuoles of various sizes (*cf.* papers in "The Biology of Hydra," Lenhoff and Loomis) which may prove to be concerned with water regulation.

It is suggestive that fresh-water hydroids as well as flatworms, coping with strongly hypotonic environments, are endowed with extensive mucus-secreting systems. A relatively thick mucous layer, perhaps merely by providing an "unstirred" external layer, is probably of critical importance in osmotic protection, although it seems unlikely that such a layer would act as a regulatory device.

It was hoped that the studies reported here would offer precise suggestions about ecological factors controlling the distribution of the hydroids, the occurrence of the three types used being quite different. The hydras in general appear limited to fresh water, though they will survive and feed in at least 5% sea water (Loomis, 1959). *Tubularia* is a typical marine form of a type not penetrating usually into the brackish waters of estuaries (Topping and Fuller, 1942).

Cordylophora appears to occur only in brackish water, not, or rarely, in the very dilute fresh water nor in full-strength ocean salt water (Roch, 1924). *Cordylophora* is the most versatile of the forms reported here since it can thrive and regenerate in a variety of salinities. Its continued growth has also been shown to be independent of any specific ion ratios, although there is a specific requirement for the presence of Na, K and Cl (Roch, 1924). Reconstitution of extruded tissue masses requires Ca or Mg and K (Beadle and Booth, 1938).

Tubularia and related forms will live and regenerate in sea water of $\frac{2}{3}$ ocean strength or less (*cf.* Keil, 1932) even though they are not usually found in such dilute habitats. A point of interest is that many marine invertebrates are reported to regenerate better in dilute than in full-strength sea water (*cf.* Keil, 1932).

With these different habitat restrictions for the hydroids, it would be good if different methods of handling ionic and osmotic problems could be demonstrated. Unfortunately, the results reported here offer no suggestions. Ionic and osmotic conditions appear to be reflected in a general pattern of distribution common to the hydroids rather than showing special variations related to habitat. A possible exception would be the presence of an Na and Cl uptake mechanism from very dilute environments. Ability of hydroids to survive in high salt media probably reflects an ability of the metabolic systems to function in high Na environments, rather than the possession of mechanisms to hold the tissue Na concentration to low levels.

The tendency to uniform tissue K concentration may be assumed to be general for all cell types of the hydroids. Thus, *Tubularia* hydranths, richly endowed with nematocysts, musculo-epithelial cells and nervous elements, have much the same electrolyte pattern as *Tubularia* stems which consist, exclusive of perisarc, of mostly ectodermal and endodermal cells without further specialization (Hyman, 1940).

LITERATURE CITED

Beadle, L. C., and F. A. Booth, 1938. The reorganization of tissue masses of *Cordylophora lacustris* and the effect of oral cone grafts with supplementary observations on *Obelia gelatinosa*. *J. Exp. Biol.*, **15**: 303–326.

Dunham, P. B., and F. M. Child, 1961. Ion regulation in *Tetrahymena*. *Biol. Bull.*, **121**: 129–140.

Fulton, C. H., 1961. The Development of *Cordylophora*. *In:* The Biology of Hydra. (H. M. Lenhoff and W. F. Loomis, eds.) University of Miami Press, Coral Gables, Florida.

Harris, E. J., 1957. Permeation and diffusion of K ions in frog muscle. *J. Gen. Physiol.*, **41**: 169–195.

Hyman, L. H., 1929. Taxonomic studies on the hydras of North America. I. General remarks and description of *Hydra americana*, new species. *Trans. Amer. Mic. Soc.*, **48**: 242–255.

Hyman, L. H., 1931. Taxonomic studies on the hydras of North America. IV. Description of three new species with a key to the known species. *Trans. Amer. Mic. Soc.*, **50**: 302–315.

Hyman, L. H., 1940. The Invertebrates. I. Protozoa through *Ctenophora*. McGraw-Hill Book Co., Inc., New York.

Keil, E. M., 1932. The effect of salts upon the regeneration of *Pennaria tiarella*. *J. Exp. Zool.*, **63**: 447–455.

Kinne, O., 1958. Adaptation to salinity variations: some facts and problems. *In:* Physiological Adaptation. (C. L. Prosser, ed.) Washington, D. C., Amer. Physiol. Soc. Pp. 92–106.

Lenhoff, H. M., and W. F. Loomis, 1961. The Biology of Hydra. University of Miami Press, Coral Gables, Florida.

Lilly, S. J., 1955. Osmoregulation and ionic regulation in *Hydra*. *J. Exp. Biol.*, **32**: 423–439.

Loomis, W. F., 1959. Control of sexual differentiation in *Hydra* by pCO_2. *Ann. N. Y. Acad. Sci.*, **77**: 73–86.

Roch, F., 1924. Experimentelle Untersuchungen an *Cordylophora caspia* (Pallas) [= *lacustris* Allman] über die Abhängigkeit ihrer geographischen Verbreitung und ihrer Wuchsformen von den physikalischchemischen Bedingungen des ungebenden Mediums. *Zeitschr. Morph. Ökologie Tiere*, **2**: 350–426.

Steinbach, H. B., 1962a. Ionic and water balance of planarians. *Biol. Bull.*, **122**: 310–319.

Steinbach, H. B., 1962b. The prevalence of K. *Perspectives in Biol. Med.*, **5**: 338–355.

Topping, F. L., and J. L. Fuller, 1942. The accommodation of some marine invertebrates to reduced osmotic pressures. *Biol. Bull.*, **82**: 372–384.

THE REGENERATION OF WHOLE POLYPS FROM ECTODERMAL FRAGMENTS OF SCYPHISTOMA LARVAE OF AURELIA AURITA [1]

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Gilchrist (1937) reported that isolated ectodermal fragments of scyphistoma larvae of *Aurelia aurita* reconstituted an entire organism. The report did not include a histological study and gave no indication of the cell type involved in the formation of the new endodermal layer. Gilchrist conjectured that interstitial cells were responsible for the formation of the new layer. In view of the uncertainty of the role of interstitial cells in hydroid regeneration and the fact that some features of reconstitution may occur in their absence (Brien *et al.*, 1953; Normandin, 1960), Gilchrist's experiments with scyphistomae of *Aurelia* were repeated and an attempt was made to trace the origin of the endoderm by means of a histological study of a staged series of reconstituting ectodermal isolates.

The observations here presented indicate that *Aurelia* scyphistomae have no cells which conform to the characteristics of typical hydroid interstitial cells. A count of mitoses reveals that somatic ectodermal cells are the most actively dividing cells and that they give rise to a population of cells (amoebocytic in appearance) which in turn become the new endodermal cells.

MATERIALS AND METHODS

Scyphistoma larvae of the marine scyphozoan, *Aurelia aurita*, were obtained through the courtesy of Dr. Sears Crowell, and were grown in the laboratory at room temperature. They were kept in small fingerbowls or plastic Petri dishes containing filtered sea water obtained from Woods Hole, Mass. The larvae were fed each morning for 30 to 60 minutes on freshly-hatched brine shrimp (*Artemia*). The *Artemia* were hatched in sea water, rinsed several times, and introduced into the cultures of *Aurelia*. After the animals had fed, the culture water was replaced with freshly filtered sea water. Water was changed again each evening.

Animals were removed from the stock culture, placed in Syracuse dishes containing filtered sea water, and starved for 24 hours prior to operation.

All operations were performed with the aid of a dissecting microscope at a magnification of either 15 × or 20 ×. Peduncle, hypostome and tentacles were removed with sharpened surgical needles. The remaining cylinder of tissue was slit

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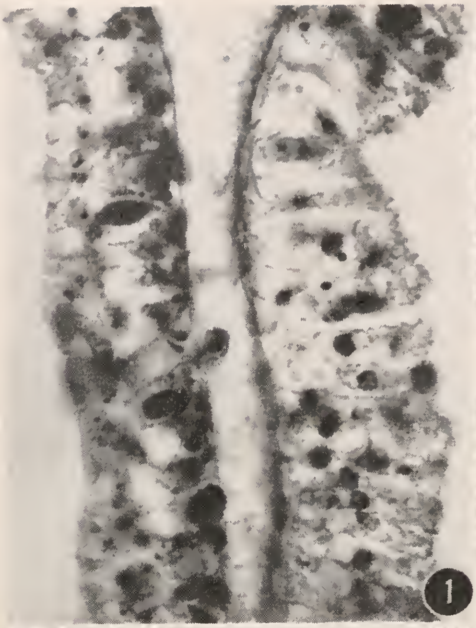

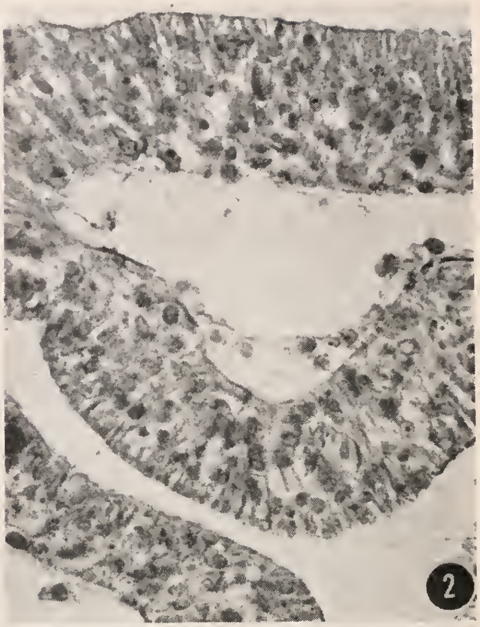

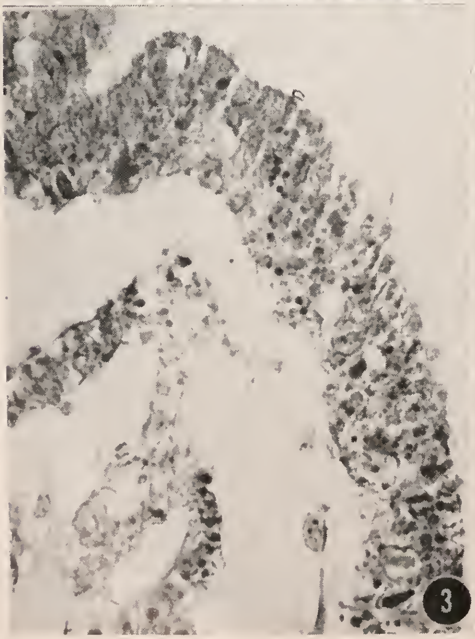

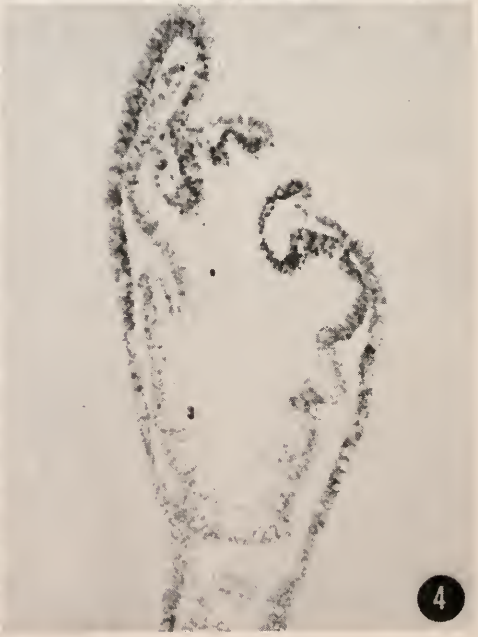

FIGURE 1. Cross-section through the mid-body region of *Aurelia*, showing ectoderm (on left), endoderm, mesoglea and an amoeboid cell. (× 840.)

FIGURE 2. Cross-section of an ectodermal fragment six hours after its separation from the endoderm, showing several amoeboid cells in the lumen. (× 520.)

FIGURE 3. Cross-section of a two-day-old regenerating ectodermal fragment. A distinct endoderm is present, and an amoeboid cell can be seen between the two layers. (× 520.)

lengthwise, flattened, and oriented so that the endodermal layer was facing upward. The tissue was then cut into six strips, each about 0.5 mm. wide, and the length of the original cylinder (about 1 mm.). The rectangular piece of tissue was held with a #5 watchmaker's forceps while a cut was made to the level of the mesoglea with a surgical needle. The endoderm was separated from the ectoderm by teasing and cutting through the mesoglea.

In *Aurelia,* the ectoderm is unpigmented, while the endoderm contains an orange pigment. This pigment is a useful diagnostic marker which helps to insure the elimination of all contaminating endoderm. The pigment marker, plus the thick mesoglea which separates the two layers, enables one to be certain of isolation of pure ectoderm. The endoderm-free isolates were transferred in a pipette to plastic Petri dishes containing filtered sea water. Fragments were sacrificed immediately after the operation (time 0), at 2, 4, 6, 8, 10, 12, 18, 20, 22, 24, 30, and 36 hours, and at 2, 3, 4, 5, and 6 days.

The specimens were fixed in one of the following fluids: Bouin's, Gilson's prepared with sea water, Carnoy's, Kleinenberg's, and Zenker's acetic. Bouin's and Gilson's were most useful, and were used in the majority of the work.

Animals were fixed for one-half to one hour, dehydrated in a graded ethanol series, cleared in cedarwood oil, and embedded in either 60–62° C. mp paraffin with 10% beeswax, or in 56–58° C. mp paraffin. Sections were cut at 7 microns.

Several stains were employed: toluidine blue (0.25% aqueous solution at pH 5), iron haematoxylin, Feulgen's, Azure B, Mallory's triple aceto-orcein. Only the first three successfully stained the sections of *Aurelia,* and they were used in most of the experiments.

RESULTS

Preliminary data

Thirty-two isolated ectodermal fragments were cultured in filtered sea water. All of these fragments formed complete hydranths. Twelve started to capture freshly hatched *Artemia* on their sixth day, the other 20 on the seventh. Ingestion of the shrimp began one day later. In addition, 41 specimens, all having a longest dimension of at least 0.18 mm., were raised until they had formed at least one tentacle. Tentacles began to appear six or seven days after isolation of the ectoderm. Eleven additional fragments, all less than 0.18 mm. long, that did not show any signs of developing tentacles by day seven, were scored as negative and were discarded.

Normal histology of Aurelia

The intact scyphistoma of *Aurelia aurita* is a two-layered, sac-like animal. In the outer, single-celled layer of ectodermal cells there are various epithelial derivatives: epitheliomuscular cells, cnidoblasts, nerve cells, columnar epithelium. The inner endodermal layer is also one cell thick. Some endodermal cells are digestive, some glandular, and others muscular. The endoderm is invaginated into the

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FIGURE 4. Longitudinal section through the mouth of a six-day-old regenerating specimen. The endodermal layer is complete, basophilic glandular cells surround the mouth, and a tentacle has formed. (× 235.)

lumen to form four longitudinal ridges, which enclose the gastric pouches. The two cell layers are separated by an acellular mesogleal layer (Fig. 1).

The base of the animal is further differentiated into a holdfast region in which the endodermal cells are enlarged and elongated. The apical region contains a mouth which is surrounded by a dense population of small, basophilic glandular cells in the endodermal layer. The mouth is ringed by a circle of tentacles, each of which bears several batteries of nematocysts.

Histology of regenerating ectodermal fragments

The development of an entire polyp from an ectodermal fragment from the mid-body region (see Fig. 1) was followed at intervals from 0 to 7 days.

(a) 0 hours: Fourteen specimens were fixed immediately after the ectoderm was separated from the endoderm. They consist of fragments of ectoderm, and some adhering mesoglea. The ectoderm appears as a single folded layer of columnar cells. Endoderm is not present in any of the sectioned specimens. An occasional large amoeboid cell is in the mesoglea of some specimens, but in no cases at a frequency of greater than 1 per 250 cells. Each explant contains a few thousand cells which are in a compact, though fragmented, mass.

(b) 2 hours: The three explants examined have lost their fragmented appearance, and have developed into a hollow mass of cells. The shape of the mass is irregular, but all of its contours are rounded. The inner margin of the ectoderm is lined with a thin layer of mesoglea.

(c) 4 hours: There is a slight increase in the number of amoeboid cells in the mesoglea of the three specimens examined. Some ectodermal cells appear to be necrotic. An occasional mitotic figure is visible in the outer margin of the columnar ectodermal layer.

(d) 6 hours: Fifteen specimens were fixed. Mitotic figures are more numerous in the ectoderm than at 4 hours, and there is an increase in the number of amoeboid cells. The latter cells are confined to the lumen, and are attached to the mesoglea. They exhibit long thin processes, are vacuolated, and have a large eccentric nucleus. No mitotic figures have been seen in them. The ectodermal cells are still all columnar, and arranged in an orderly fashion, similar to that observed in the normal animal (Fig. 2).

In some areas there is a necrotic mass containing many refractile inclusions and semi-lunar, Feulgen-positive bodies. The refractile bodies appear to be breakdown products of cnidoblasts. Mitotic activity is not found in the area surrounding the necrotic masses, and amoeboid cells are rare in this region.

(e) 8 hours (3 specimens): There are areas in which the lumen is obliterated by necrotic masses. In non-necrotic portions of the animal there are invaginations of ectoderm; the population of amoeboid cells is most dense in these regions of invagination.

(f) 10 hours (3 specimens): The lumen is large, and the areas of necrosis are less numerous. The number of amoeboid cells is about the same as at six hours.

(g) 12 hours (14 specimens): Mitotic activity is still confined to the ectoderm, and is occurring at a surprisingly high rate of about 1/250 cells. There is a marked increase in the amount of mesoglea.

(h) 18 hours (11 specimens): The mitotic rate is more than double that seen at 12 hours. Mitotic activity is confined almost exclusively to the ectoderm; in all of the sections studied only one amoeboid cell was seen in the process of division.

(i) 20 hours (3 specimens): No apparent change is visible.

(j) 22 hours (3 specimens): Amoeboid cells are numerous, and are found scattered throughout the lumen. Several are packed densely near the point of invagination of the ectoderm. The ectoderm is now extremely regular, with the long axis of each cell pointing to the center of the enclosed area and the nuclei all basal. Cnidoblasts have appeared.

(k) 24 hours (18 specimens): Some areas of the lumen are densely populated with amoeboid cells. The mitotic rate has not changed noticeably since 18 hours, and mitotic activity is still confined to the ectoderm.

(l) 30 hours (2 specimens): The amoeboid cells are beginning to line up end to end, or to arrange themselves into small groups. They are confined to the lumen, and are visible on both sides of the mesogleal layer, with which they are in contact. Some cells have extended long filamentous processes toward neighboring cells, and are in contact with these neighbors via the resulting bridges. There is little mitotic activity. Necrotic areas are no longer visible.

(m) 36 hours (2 specimens): The amoeboid cells are lined up in tandem, and form a distinct second layer, in which some typical endodermal cells may be seen for the first time.

(n) 2 days (11 specimens): There are two distinct layers of cells; the outer is ectodermal, the inner is endodermal. Mitotic activity is confined to the ectoderm and is becoming more frequent (Fig. 3).

(o) 3 days (13 specimens): Endodermal mitoses are becoming apparent, but are less frequent than ectodermal mitoses. An endodermal layer is distinct and, with the exception of one specimen, amoeboid cells are very rare. The exceptional specimen has one area with abundant amoeboid cells and no endodermal layer. Except for the one area this specimen has a distinct endodermal layer lacking amoeboid cells.

In the other specimens at this time the endoderm is a distinct, coherent layer, and, in usual fashion, is separated from the ectoderm by the mesoglea. A mouth is beginning to form at one end of the mass.

(p) 4 days (12 specimens): There is little change from three days. Amoeboid cells are rare in all specimens.

(q) 5 days (6 specimens): The animals are forming apical outpocketings (consisting of both ectoderm and endoderm) in the region of the hypostome. There are invaginations of the endoderm which resemble gastric pouches.

(r) 6 days (3 specimens): The mouth has formed, and the area of the hypostome is surrounded by small, basophilic glandular cells. At the opposite end of the animal, the endodermal cells have enlarged into typical vacuolated stalk cells. Tentacles, complete with batteries of nematocysts, are present, although they vary in number and size from specimen to specimen. Mitotic activity is found in both ectoderm and endoderm (Fig. 4).

(s) 7–10 days: Some time during this period the regenerant begins to feed. Several specimens, including at least one from each set of experiments, have been followed for a few weeks, until they bud off a new scyphistoma.

DISCUSSION

Gilchrist (1937) assumed, with no microscopic evidence to support his contention, that the newly formed endodermal cells were formed from interstitial cells in his ectodermal isolates of *Aurelia*. The present study has failed to reveal typical interstitial cells in either an intact scyphistoma, or during any stage of reconstitution. During regeneration, large amoeboid cells, similar to ones seen only infrequently in the intact animal, begin to appear. These cells do not have the typical basophilic character of a standard interstitial cell; instead, they are undistinguished cells, with no definite shape or characteristic cytoplasmic constitution.

These amoeboid cells could have come from either pre-existing amoeboid cells or from somatic ectoderm cells. The evidence indicates that the latter is the case. This evidence is based on fourteen ectodermal isolates which were selected at random during the experiments and fixed immediately after preparation. Nine of these contained no amoeboid cells; the other five had such cells but in no case was there more than one for every 250 ectoderm cells. Since all isolates of pure ectoderm which were larger than 0.18 mm. in length regenerated completely, we can assume (if the fourteen isolates are representative) that initial presence of amoeboid cells is not required for formation of an endodermal layer. Further, there was a consistent increase in the relative number of amoeboid cells with time after isolation, and such cells were found in all specimens fixed four hours or longer after preparation. Since this increase occurred in the absence of significant mitotic activity in the amoeboid cell population and was accompanied by a high mitotic rate of the ectoderm, we are led to the conclusion that the amoeboid cells, which eventually form the endodermal cells, are derived from the somatic ectoderm cells.

The source of cells in regeneration has not been clearly established. Attempts to link the new cells to interstitial cells or neoblasts often involve x-irradiation. Such treatment inhibits migration of interstitial cells, and regeneration ceases (Brien and Reniers-Decoen, 1955; Burnett, 1961; Puckett, 1936). One can conclude that interstitial cells are necessary for regeneration only if one assumes that x-irradiation does not affect somatic cells. Most studies of regeneration involve cases where all somatic cell types are represented. These are known to divide, and can be the source of the new cells. The question at issue is whether a somatic cell of one type is capable, regardless of its pathway, of forming a cell of another type. It has been proposed that, once a cell has received and used the information which determines its adult type, it loses its capacity to become another type of cell. The situation can best be assessed where one tissue type is eliminated. This has been done in the present study. *Aurelia* possesses no reserve cells, and in the present study it has been shown to be capable of reconstituting an endodermal layer when this layer is absent. The evidence is strong that somatic ectoderm can give rise to somatic endoderm, by first losing its characteristics and becoming an "indifferent" amoeboid cell.

SUMMARY

1. Isolated ectodermal fragments of *Aurelia aurita* scyphistomae regenerate into complete hydranths. *Aurelia* possesses no interstitial cells, but large amoeboid cells appear during reconstitution.

2. The endoderm forms by differentiation of the amoeboid cells, which have probably arisen by dedifferentiation of the ectoderm.

LITERATURE CITED

Brien, P., and J. P. van Den Eeckhoudt, 1953. Bourgeonnement et régénération chez les Hydres irradiées par les rayons X. *C. R. Acad. Sci.*, **237**: 756–758.

Brien, P., and M. Reniers-Decoen, 1955. La signification des cellules interstitielles des hydres d'eau douce et le problème de la réserve embryonnaire. *Bull. Biol. France Belgique*, **89**: 258–325.

Burnett, A. I., 1961. The growth process in *Hydra*. *J. Exp. Zool.*, **146**: 21–83.

Gilchrist, F. G., 1937. Budding and locomotion in the scyphistomas of *Aurelia*. *Biol. Bull.*, **72**: 99–124.

Normandin, D. K., 1960. Regeneration of *Hydra* from the endoderm. *Science*, **132**: 678.

Puckett, W. O., 1936. The effects of x-radiation on the regeneration of the hydroid, *Pennaria tiarella*. *Biol. Bull.*, **70**: 392–399.

MARINE

CUTANEOUS AND PULMONARY GAS EXCHANGE IN THE SPOTTED SALAMANDER, AMBYSTOMA MACULATUM[1]

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The first quantitative study of pulmonary and cutaneous respiration in amphibians was conducted on *Rana esculenta* and *R. fusca* by Krogh (1904). He inserted a cannula, connected to an air pump, into the trachea and analyzed separately the air forced through the lungs by the pump and the air surrounding the frog. Krogh found that carbon dioxide was released chiefly through the skin, while oxygen was taken up predominantly by the lungs. He also found that oxygen uptake through the skin remained relatively constant throughout the year, while oxygen uptake by the lungs was greatest during the spring and dropped below cutaneous uptake during the fall and winter. His curves for release of carbon dioxide through the skin and lungs followed the pulmonary oxygen uptake curve throughout the year. Dolk and Postma (1927), using similar techniques with *Rana temporaria*, substantiated Krogh's results.

Lapicque and Petetin (1910) demonstrated that cutaneous respiration in the lungless salamander, *Euproctus montanus*, may be more important than lung and/or buccopharyngeal respiration. They found that *E. montanus* dies quickly when submerged in Vaseline with its head free, but can live without buccopharyngeal respiration. However, their study did not solve the problem of the relative importance of cutaneous and buccopharyngeal or pulmonary respiration in salamanders.

Since the relative roles of pulmonary and cutaneous respiration in salamanders had not been studied quantitatively, we undertook the present study to determine the role of each in the respiration of *Ambystoma maculatum*.

METHODS AND MATERIALS

The animals used in this study were collected in late March, 1962, in the vicinity of Kingston, R. I. Groups of animals were acclimated in constant temperature chambers in total darkness. The minimum standards used for acclimation were as follows:

5° C. - - - one week at 15° C.; two weeks at 10° C.; 10 days at 5° C.
10° C. - - - one week at 15° C.; two weeks at 10° C.
15° C. - - - one week at 15° C.
25° C. - - - one week at 15° C.; one week at 25° C.
30° C. - - - one week at 15° C.; one week at 25° C.; 5 days at 30° C.

A mask of 0.5-inch Tygon flexible plastic tubing was sutured to the head of the animal at least 24 hours prior to use. The mask was constructed in a manner that

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would not interfere with normal buccal movements (Fig. 1). Pulmonary and cutaneous respiration were determined in a closed system respirometer.

The respirometer consisted of four chambers of equal volume constructed of 0.25-inch acrylic plastic (Fig. 2). The two rear chambers served as thermobarometers, while pulmonary and cutaneous respiration were measured separately and simultaneously in the two front chambers, which were connected by a 0.5-inch hole. A cover of 0.25-inch plastic was screwed down over the four chambers and sealed with petroleum jelly. A series of plastic connectors with stopcocks communicated through the cover into the respiration chambers. Manometers with colored kerosene indicators were fitted into the stopcock connectors. Syringes filled with 100% oxygen were fitted to the stopcock connectors of the pulmonary and cutaneous chambers. All connections were checked for leaks prior to each set of experiments.

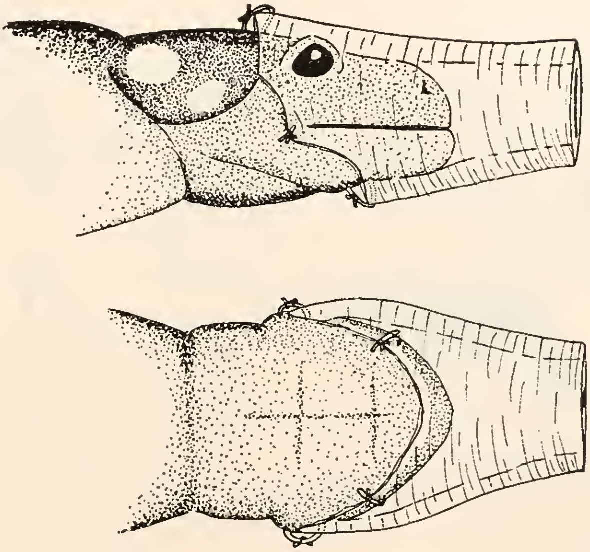

FIGURE 1. Method of attaching plastic mask to salamanders.

The respirometer was constructed within a larger plastic chamber which served as a water jacket. A cooling coil, heating coil, temperature regulator, and stirrer were placed in the water jacket for temperature control. The temperature-regulating system kept the water temperature constant within ±0.1° C.

The masked animal was tied firmly to a piece of hardware cloth and the mask fitted through the hole between the chambers. The end of the mask was sealed into the hole in the chamber wall by the application of petroleum jelly. Beakers containing 10 ml. of barium hydroxide were placed in each chamber to absorb carbon dioxide. The beakers of barium hydroxide contained plastic-coated magnetic bars which were moved at regular intervals by a magnet outside the respirometer chambers. This stirred the barium hydroxide solution and insured an effective absorption of the carbon dioxide by breaking the barium carbonate film which formed on the surface and which would have resulted in reduced absorption of carbon dioxide. Oxygen injected into the chambers by the syringes compensated for oxygen consumed by the animal. Oxygen consumption was read directly from the calibrated syringes.

At the end of a set of experiments, the beakers of barium hydroxide were removed from the chambers and titrated with standardized 1 N sulfuric acid to determine the quantity of carbon dioxide produced. The beakers of barium hydroxide in the thermobarometers served as controls, since each beaker of barium hydroxide absorbed carbon dioxide at the same rate both prior to the experiment and during the time required for titration. To determine the actual amounts of carbon dioxide released by the animal, the amount of carbon dioxide absorbed in the thermobarometers was subtracted from the amounts of carbon dioxide absorbed in the pulmonary and cutaneous chambers.

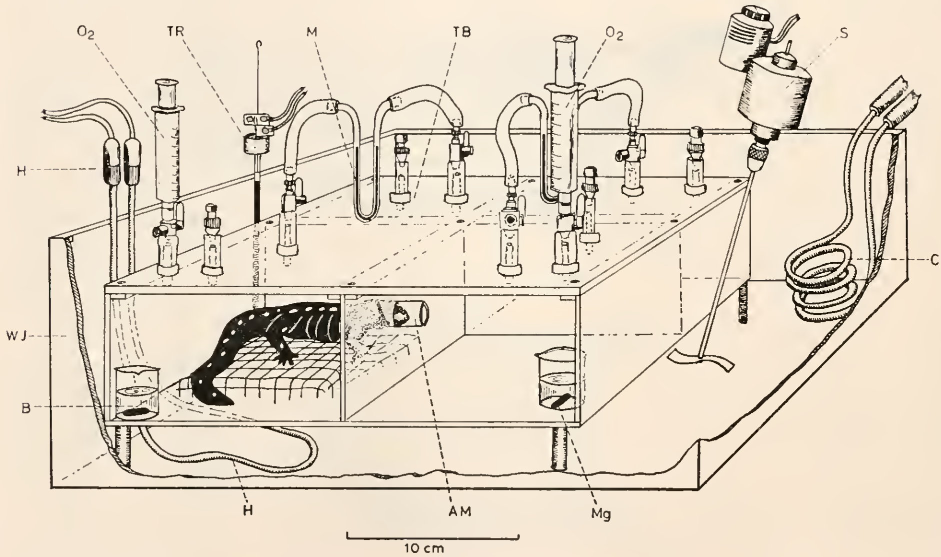

FIGURE 2. Apparatus used to measure simultaneously pulmonary and cutaneous respiration in amphibians. AM, animal mask; B, barium hydroxide solution in beaker; C, cooling coil; H, heating coil; M, manometer; Mg, magnetic stirring bar; O_2, oxygen syringe; TB, thermobarometer chamber; TR, temperature regulator; S, stirrer; WJ, water jacket.

The few animals that struggled against their bonds during the first hour of the experiment produced high oxygen consumption values. The values obtained for these hours were not included in the calculations of mean oxygen consumption, but the carbon dioxide produced had to be included in the determination of respiratory quotients, since the barium hydroxide could not be removed for titration after each hour.

Measurements of total oxygen consumption were made as controls. Differences in oxygen consumption between masked and unmasked animals were not statistically significant. Oxygen consumption measurements for masked and unmasked animals tied to hardware cloth indicated that restraint of the animals resulted in a slight increase in oxygen consumption. Total oxygen consumption for experimental animals was about 5% higher than unrestrained controls at all temperatures except 5° C., where movement was negligible. Respiratory quotient values for control animals were not significantly different from those of experimentals.

Tidal volumes were measured by connecting the animal's mask to a graduated manometer. The volume of air required to move the manometer column a distance equal to that moved by the breathing of the animal was taken as the tidal volume.

Recordings of breathing movements were obtained for masked and unmasked animals acclimated to 10° C., 15° C., and 25° C. by passing a loop of thread under the buccal floor of the animal and connecting it to a pressure transducer. Movement of the buccal floor caused movement of the transducer wire, which was connected to a Physiograph.

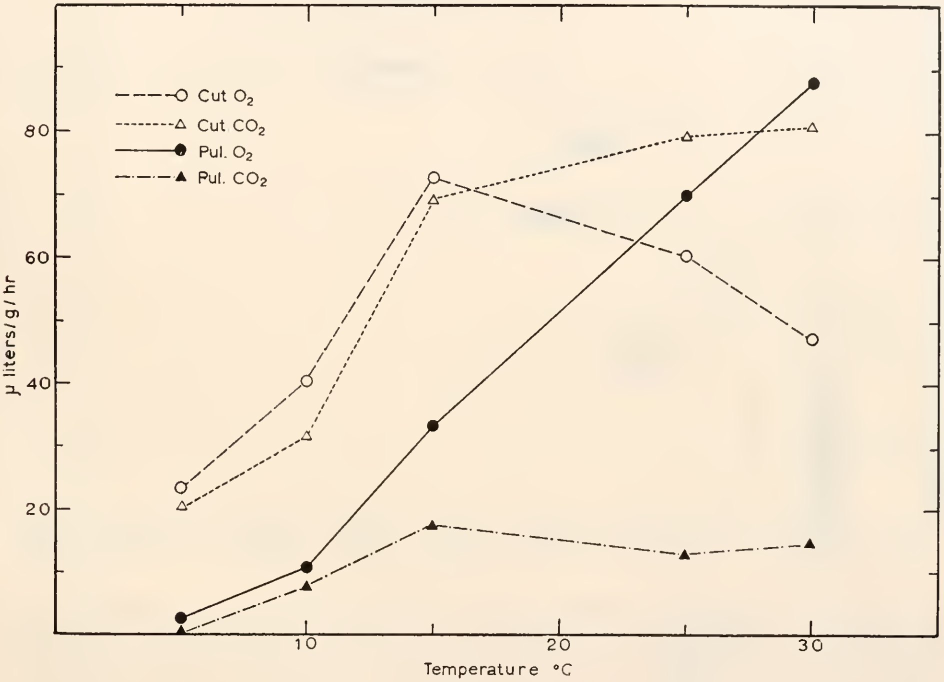

Figure 3. Mean cutaneous and pulmonary gas exchange at different temperatures.

Results

Pulmonary and cutaneous gas exchange

Pulmonary oxygen consumption increased almost linearly from 1.36 μl./gm./hr. at 5° C. to 86.59 μl./gm./hr. at 30° C. Cutaneous oxygen increased from a mean of 22.74 μl./gm./hr. at 5° C. to 73.00 μl./gm./hr. at 15° C., then dropped to 46.62 μl./gm./hr. at 30° C. (Figs. 3 and 4). The ratio of pulmonary to cutaneous

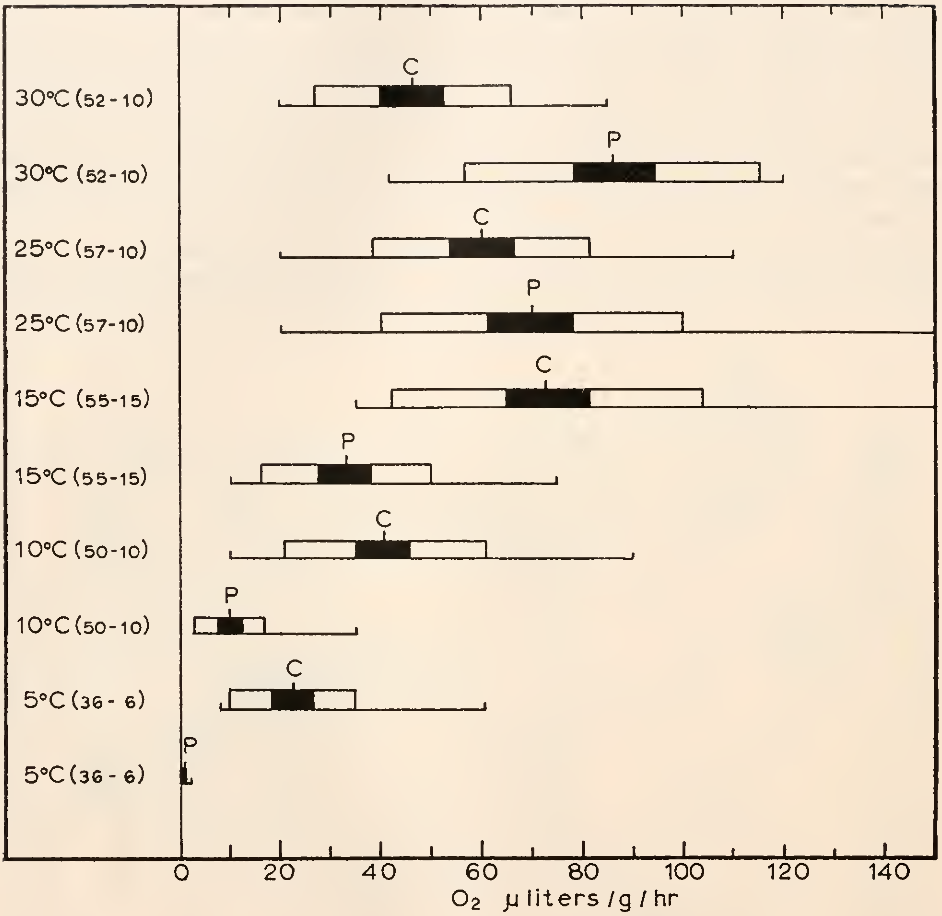

FIGURE 4. Cutaneous and pulmonary oxygen consumption at different temperatures. The first figure in parentheses indicates total number of hours of measurement; the second figure denotes number of individuals in sample. The horizontal line indicates the range, the thin vertical line, the mean; one black and one white rectangle combined on each side of the mean, one standard deviation; one black rectangle on each side of the mean, two standard errors. If the standard errors of two sets of data do not overlap, the difference between the means may be considered statistically significant (Hubbs and Hubbs, 1953).

oxygen consumption increased with temperature (Fig. 5). No measureable amount of carbon dioxide was released through the lungs and buccopharyngeal mucosa at 5° C. A sharp increase in both pulmonary and cutaneous carbon dioxide occurred between 10° and 15° C. At temperatures above 15° C. pulmonary carbon dioxide remained almost constant (Figs. 3 and 6). Cutaneous carbon dioxide increased gradually with increasing temperature. The ratio of pulmonary to cutaneous carbon dioxide release was approximately 0.2 at all temperatures except at 5° C., where there was no measureable release of carbon dioxide from the lungs and buccopharyngeal surfaces.

Respiratory quotients

The range and mean of respiratory quotients, (RQ), were: 5° C. 0.73–0.81, $\bar{x} = 0.76$; 10° C., 0.71–0.85, $\bar{x} = 0.77$; 15° C., 0.72–0.89, $\bar{x} = 0.78$; 25° C., 0.70–0.87, $\bar{x} = 0.76$; 30° C., 0.71–0.88, $\bar{x} = 0.77$. Animals in acclimation were fed a diet of mealworms at regular intervals; those that refused to eat had RQ values between 0.70 and 0.72. In animals kept at 30° C., the mean RQ changed from 0.82 one to three days after feeding, to 0.72 five days after feeding. For animals acclimated to 10°, 15°, and 25° C., RQ's between 0.84 and 0.80, observed

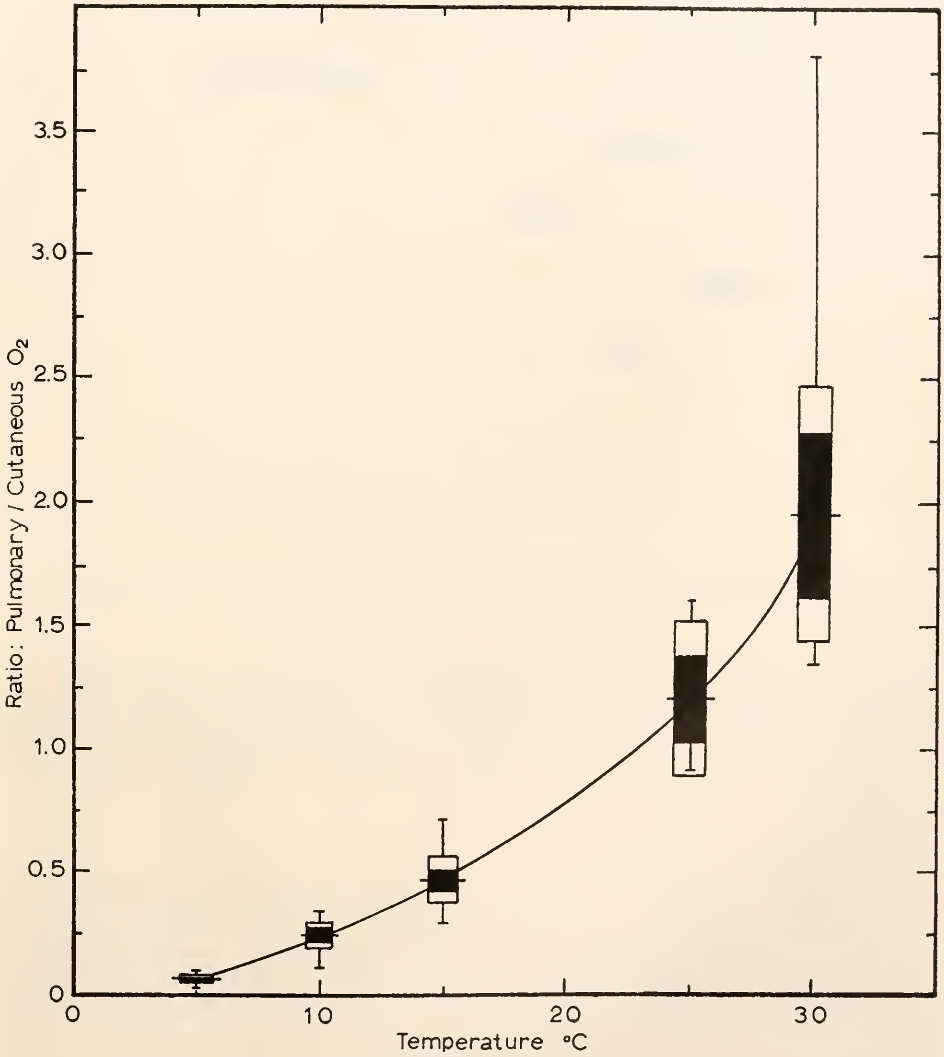

FIGURE 5. The ratio of pulmonary to cutaneous oxygen consumption at different temperatures. Method of presentation is the same as in Figure 4.

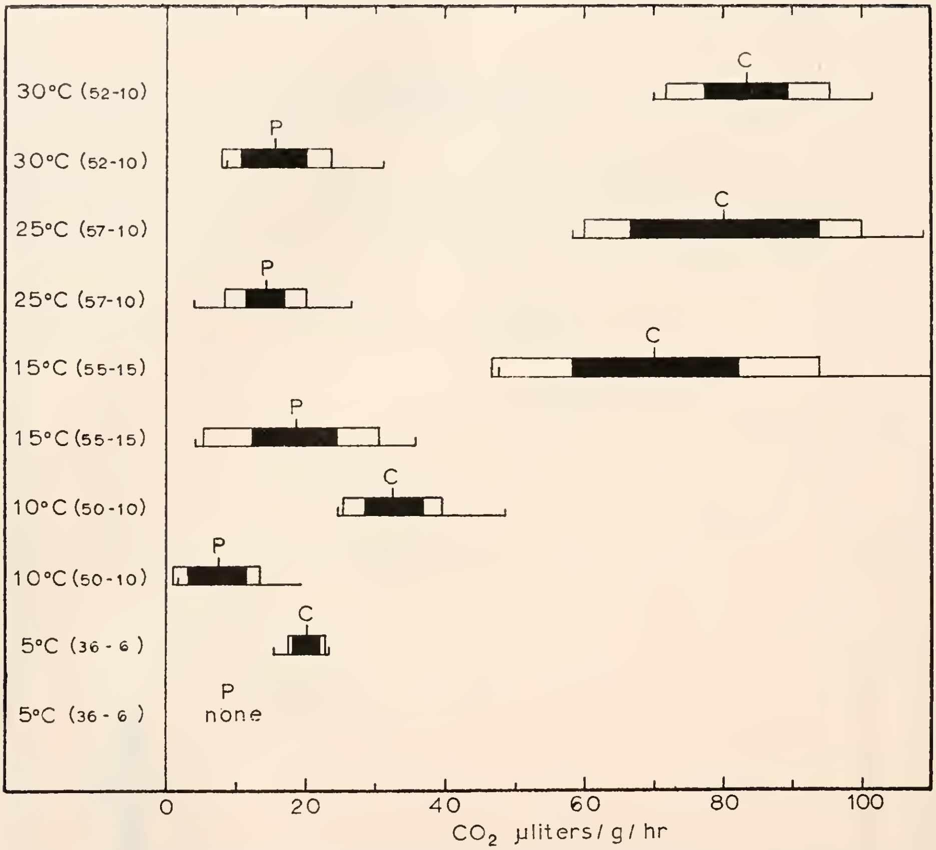

Figure 6. Cutaneous and pulmonary carbon dioxide release at different temperatures. Method of presentation is the same as in Figure 4.

from one to four days after feeding, decreased to 0.72 eight days after feeding. At 5° C., mean RQ's increased from 0.74 three days after feeding, to 0.78 five days after feeding. Meal worms could be detected in the stomachs of animals acclimated to 5° C., up to five days after feeding, indicating incomplete digestion and assimilation.

Tidal volumes and breathing rates

Two distinct breathing movements, including two separate tidal volumes, occur in salamanders with lungs. The buccopharyngeal movement consists of an enlargement of the buccopharyngeal cavity by a lowering of the hyobranchial apparatus, resulting in the inspiration of air through the nares. Exhalation occurs when the buccal floor rises again. A pronounced depression of the buccal floor occurs at intervals. During the latter part of this depression, the nares completely close, the buccal floor raises and forces the air into the lungs (Whipple, 1906).

Temperature had a direct effect on tidal volumes (Fig. 7). The mean buccopharyngeal tidal volume increased from 0.008 cc. at 5° C. to 0.065 cc. at 25° C. and 30° C. The mean lung tidal volume increased from 0.09 cc. at 5° C. to 0.42 cc. at 30° C.

The number of deep inspirations, *i.e.*, those movements in which air was forced into the lungs, remained relatively constant (between six and nine per minute at

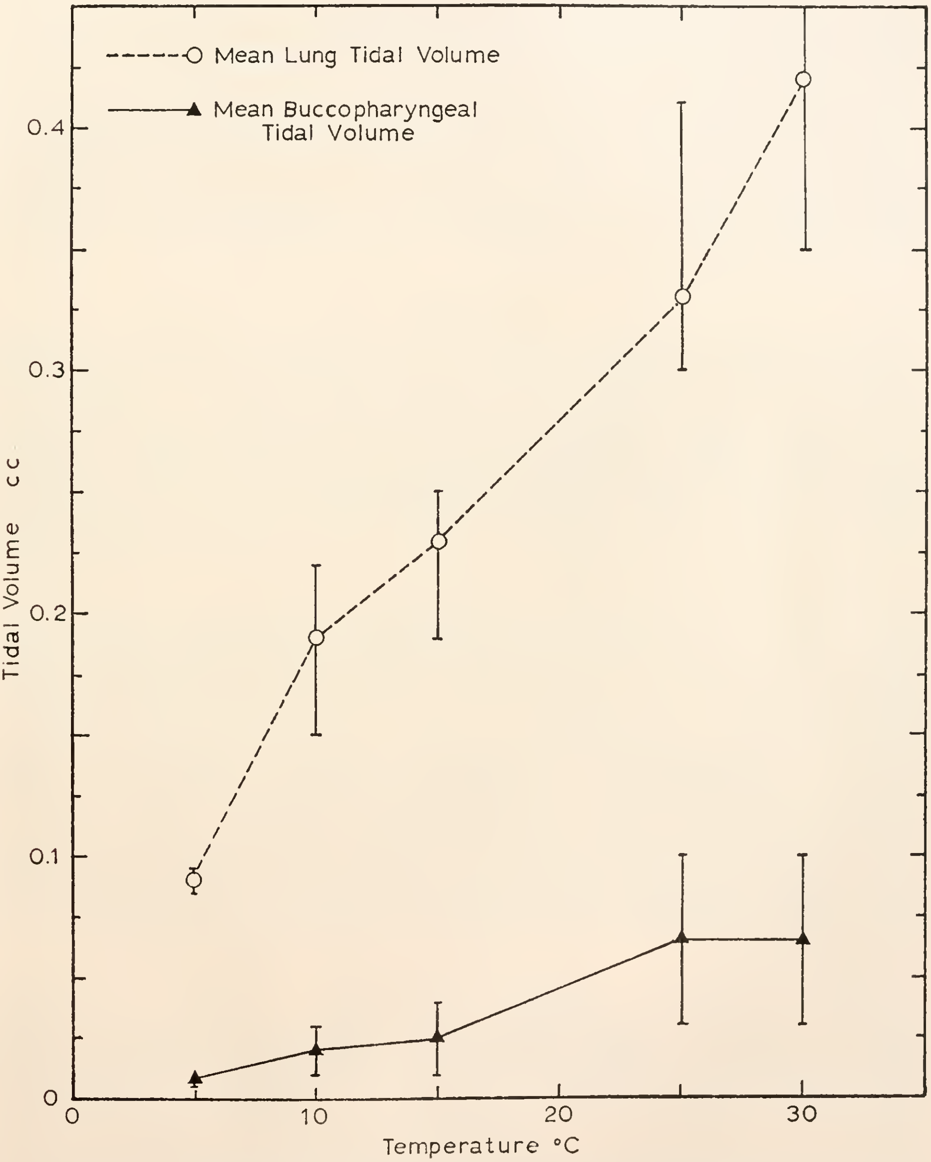

Figure 7. Mean lung and buccopharyngeal tidal volumes at different temperatures. Vertical lines denote the range of observed variation.

all temperatures). However, the rate of buccopharyngeal oscillations was dependent upon temperature. The average rate of buccopharyngeal movements was 13 at 10° C., 80 at 15° C., and 91 at 25° C. Masking had no visible effect on breathing rates.

The mean volume of air inspired by buccopharyngeal movements at 10° C., was 15.6 cc. per minute; by lung inspirations, 68.4 cc. per minute; but at 25° C. the mean volume of air inspired by buccopharyngeal movements was 382.2 cc. per minute and by lung inspirations, 194.4 cc. per minute. Thus, the volume of air moved by buccopharyngeal oscillations at 25° C. is approximately 25 times that moved at 10° C., while that moved by the lungs is only about three times as great.

The per cent efficiency of the lung-buccopharyngeal mucosa combination was calculated by dividing the oxygen consumption per hour by the total amount of oxygen inspired, the total volume of inspired air being calculated from breathing rates and tidal volumes. The following are the calculated efficiencies: 10° C., 1.43%; 15° C., 0.93%; 25° C., 0.86%.

Discussion

Krogh (1904) and Dolk and Postma (1927) probably introduced experimental error into their measurements of lung respiration by failure to acclimate their experimental animals adequately to constant temperature. In addition, they cannulated the trachea and forced air in and out of the lungs with a mechanical pump, a procedure that probably does not duplicate the two distinct breathing movements in frogs (Cole and Allison, 1929; Scholten, 1942). The mask used in our experiments did not affect either the breathing movements or the breathing rates of the experimental animals.

With increasing environmental temperatures, the lungs and buccopharyngeal mucosa play an increasing role in oxygen uptake in *A. maculatum*. Since oxygen uptake through the skin is passive, it is dependent upon the proximity of the capillaries to the surface of the skin, the rate of blood flow through the capillaries, and the affinity of the hemoglobin for oxygen. Uptake of oxygen through the lungs and buccopharyngeal mucosa is not only dependent on these same factors but also on the depth and rate of breathing movements. Therefore, the increase in oxygen uptake through the lungs and buccopharyngeal mucosa that occurs with rising temperature can be directly correlated with increases in tidal volumes and in breathing rates. At the same time, the rate of lung inspirations remains relatively constant, changing from six at 10° C. to nine at 25° C., while the rate of buccopharyngeal oscillations increases greatly, from 13 per minute at 10° C. to 91 per minute at 25° C.

Matthes (1927), Vos (1936) and Elkan (1955) concluded that the buccopharyngeal oscillations of amphibians were olfactory in function, while Noble (1925) had assumed that these movements were primarily for respiration. Czopek (1962) found that the capillaries of the mouth cavity in *A. opacum* accounted for only 6.4% of the respiratory capillaries and concluded that (p. 586), "the pulsation of the buccal floor . . . is probably connected with olfactory functions rather than respiration." He pointed out, however, that (p. 586) "conclusions derived exclusively from morphological findings must be accepted with prudence unless they are supported by physiological investigations." Our data indicate that buccopharyngeal movements are of appreciable value in respiration, especially at higher temperatures. Between 10° C. and 25° C., the volume of air moved through the lungs increases about three-fold; the volume of air moved through the buccopharyngeal cavity increases 25-fold. If the air moved by the buccopharyngeal oscillations were excluded from respiration, the efficiency of the lungs would have to double to account for the increased oxygen consumption at 25° C.

In nature during the warmer months of the year, *A. maculatum* would probably have food in its stomach three to five days after feeding. At normal environmental temperatures (10° C. to 25° C.), five to six days are necessary for complete digestion and assimilation of food because during this period RQ values remain above fasting levels.

Cutaneous oxygen uptake increased linearly between 5° C. and 15° C., but dropped to lower values at 25° C. and 30° C. This decrease in oxygen uptake through the skin at temperatures above 15° C. may be due to several factors. *A. maculatum,* in its natural environment, remains burrowed in moist leaf litter or rotten logs, coming to the surface to feed at night, and is most active on rainy nights. In this micro-environment, this species rarely encounters temperatures exceeding 20° C. It is possible, therefore, that during its evolutionary history, certain enzymes or other physiological systems became adapted to function optimally at temperatures approximating 15° C. to the point where they are not sufficiently labile to be altered significantly by changes in acclimation temperatures. If enzyme systems of *A. maculatum* are adjusted to function optimally at temperatures approximating 15° C., higher temperatures could result in decreased oxygen uptake through the skin.

SUMMARY

1. In *Ambystoma maculatum,* the lungs and buccopharyngeal mucosa become increasingly important in respiration at higher temperature.

2. The skin accounts for more than 50% of the total oxygen uptake at 15° C. and below.

3. Approximately 80% of the carbon dioxide produced is released through the skin at all temperatures except 5° C., where no measureable amount of carbon dioxide is released through the lungs and buccopharyngeal mucosa.

4. Lung and buccopharyngeal tidal volumes increased directly with temperature; and the rate of buccopharyngeal oscillations increased greatly at higher temperatures, while the rate of lung inspirations remained relatively constant.

5. Buccopharyngeal oscillations are of appreciable importance in the respiration of *A. maculatum,* especially at higher temperatures.

LITERATURE CITED

COLE, W. H., AND J. B. ALLISON, 1929. The pharyngeal breathing rate of the frog as related to temperature and other factors. *J. Exp. Zool.,* **53**: 411–420.

CZOPEK, J., 1962. Vascularization of respiratory surfaces in some Caudata. *Copeia,* **1962**: 576–587.

DOLK, H. E., AND N. POSTMA, 1927. Über die Haut und die Lungenatmung von *Rana temporaria. Zeitschr. vergl. Physiol.,* **5**: 417–444.

ELKAN, E., 1955. The buccal and pharyngeal mucous membrane in urodeles. *Proc. Zool. Soc. London,* **125**: 685–692.

HUBBS, C. L., AND CLARK HUBBS, 1953. An improved graphical analysis and comparison of series of samples. *Syst. Zool.,* **2**: 49–57.

KROGH, A., 1904. On the cutaneous and pulmonary respiration of the frog. *Skand. Arch. Physiol.,* **15**: 328–419.

LAPICQUE, L., AND J. PETETIN, 1910. Sur la respiration d'un batracien urodele sans poumons, *Euproctus montanus. C. R. Soc. Biol.,* **69**: 84–86.

MATTHES, E., 1927. Der Einfluss des Mediumwechsels aus das Geruchsvermögen von Triton. *Zeitschr. vergl. Physiol.,* **5**: 83–166.

NOBLE, G. K., 1925. The integumentary, pulmonary and cardiac modifications correlated with increased cutaneous respiration in the Amphibia; a solution of the "hairy frog" problem. *J. Morph. Physiol.,* **40**: 341–416.

SCHOLTEN, J. M., 1942. A few remarks on the respiratory movements of the frog. *Arch. Néerl. Sci.,* **26**: 250–268.

VOS, H. I., 1936. Über die Atembewegungen und den Schnuffelmechanismus (Kehloszillationen) bei Reptilein und Amphibien. *Zool. Anz.,* **115**: 142–144.

WHIPPLE, I. L., 1906. The ypsiloid apparatus of urodeles. *Biol. Bull.,* **10**: 255–297.

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THE JUVENILE HORMONE. III. ITS ACCUMULATION AND STORAGE IN THE ABDOMENS OF CERTAIN MALE MOTHS

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In the previous papers of this series (Williams, 1959, 1961) a simple and highly selective test for the juvenile hormone was described in terms of its ability to suppress the transformation of pupae into adult moths. By the use of this so-called "pupal assay" the role of the juvenile hormone was investigated during successive stages of the metamorphosis of the Cecropia silkworm.

A by-product of these studies was a finding which one could scarcely have predicted by any rational process; namely, that the abdomens of male Cecropia and Cynthia moths contain a cache of juvenile hormone. The object of the present communication is to document this fact in experiments performed on five species of wild silkworms—Cecropia, Cynthia, Polyphemus, Pernyi, and Orizaba.

METHODS

In addition to methods previously described (Williams, 1959, 1961), the following special procedures were utilized:

1. *Parabiosis between pupae and headless moths*

The moth was deeply anesthetized with carbon dioxide. The antennae and the fore and midlegs were excised and melted wax was applied with a drawing-pen to cover the entire head and prothorax. Then, with a single transverse cut, the head was removed to leave a collar of wax at the anterior open end of the prothorax. Crystals of an equal-part mixture of phenylthiourea and streptomycin sulfate were placed in the wound along with enough Ringer's solution to fill the cavity.

The pupal partner was deeply anesthetized with carbon dioxide and a disc of integument, about 4 mm. in diameter, was cut from the mesothoracic tergum. The underlying epidermis was trimmed away with microscissors, care being taken to avoid any damage to the aorta which extends beneath the midline at this point. Melted wax was applied to the integument around the margin of the wound. Crystals of phenylthiourea and streptomycin were placed in the wound and the cavity was filled with a few drops of Ringer's solution.

The two animals were oriented in a cradle of plasticene and the wax-coated openings were brought into juxtaposition. The pupal abdomen was compressed until the pupal blood filled the narrow opening between the animals, all air being thereby displaced. The junction was then sealed with melted wax to yield a parabiotic preparation such as shown in Figure 1.

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The preparation was then removed from the anesthesia funnel and stored at 25° C. Under this condition, the moth commonly initiated energetic flapping of its wings. In order to prohibit this activity, the wings were placed between the jaws of a spring-loaded clothes-pin.

2. *Parabiosis between pupae and adult abdomens*

Melted wax was applied to the first abdominal segment of an anesthetized moth. With a single transverse scissor-cut the entire abdomen was detached from the thorax. The wax-coated edges of the wound were spread apart and the glistening air-filled crop was grasped with forceps and removed. Crystals of the phenylthiourea-streptomycin mixture were placed in the wound along with a few drops of Ringer's solution. The adult abdomen was then joined in parabiosis with a pupal partner, as described above. A preparation of this type is shown in Figure 3.

TABLE I

Parabiosis between diapausing Cecropia pupae and headless moths

Species of moth		Sex of pupal partner	Number of preparations	Results
Polyphemus	♀	♀	2	Prolonged survival* but no development
	♀	♂	2	Prolonged survival but no development
	♂	♀	2	Prolonged survival but no development
	♂	♂	2	Prolonged survival but no development
Pernyi	♀	♀	2	Prolonged survival but no development
	♀	♂	2	Prolonged survival but no development
	♂	♀	2	Prolonged survival but no development
	♂	♂	2	Prolonged survival but no development
Cecropia	♀	♀	2	Prolonged survival but no development
	♀	♂	2	Prolonged survival but no development
	♂	♀	5	Prolonged survival but no development (2) Pupae developed into moths retaining many pupal characters (3)
	♂	♂	4	Prolonged survival but no development (3) Pupa developed into moth retaining many pupal characters (1)

* Moths survived for up to 10 weeks; pupae survived up to 6 months.

RESULTS

1. *Parabiosis between diapausing Cecropia pupae and headless moths*

Thirty-five diapausing Cecropia pupae were joined in parabiosis with headless Polyphemus, Pernyi, and Cecropia moths. Six of the preparations died within a week at 25° C. and were discarded. The behavior of the 29 viable preparations is outlined in Table I.

There are three points of interest in this table. The first is the spectacular prolongation of life in moths joined to pupal partners. The second point of interest is the lack of any developmental response in the vast majority of preparations. But the most surprising finding of all is the fact that 4 of 9 diapausing pupae initiated development when joined to headless male Cecropia moths; moreover, in each of these cases the pupa developed and molted into an adult which preserved numerous pupal characteristics throughout head, thorax, and abdomen. These four pupae, in short, behaved as if they had been implanted with active corpora allata (Williams 1952a, 1959, 1961).

TABLE II

Parabiosis between previously chilled pupae and headless moths

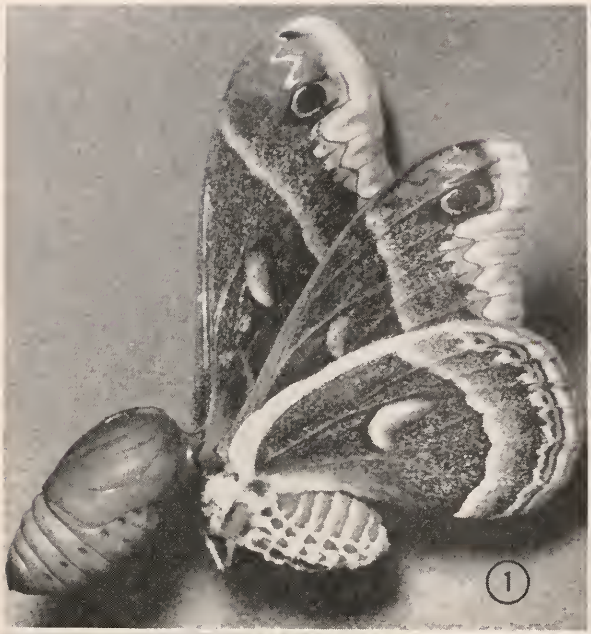
Species of moth	Species of pupal partners	Number of preparations	Effects on pupal partner
♀ Polyphemus	♂ or ♀ Polyphemus	4	Formed normal moths
♂ Polyphemus	♂ or ♀ Polyphemus	4	Formed normal moths
	♂ or ♀ Cecropia	4	Formed normal moths
♀ Cecropia	♂ or ♀ Polyphemus	4	Formed normal moths
	♂ or ♀ Cecropia	4	3 formed normal moths 1 formed moth retaining a patch of pupal cuticle on thoracic tergum
♂ Cecropia	♂ or ♀ Polyphemus	2	2 developed into moths retaining many pupal characters
	♂ or ♀ Cecropia	8	8 developed into moths retaining many pupal characters

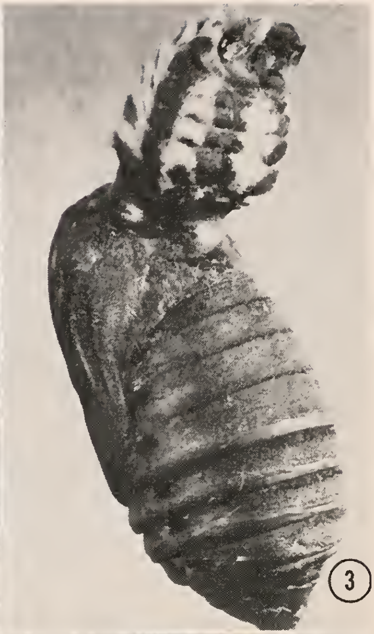
2. *Parabiosis between previously chilled pupae and headless moths*

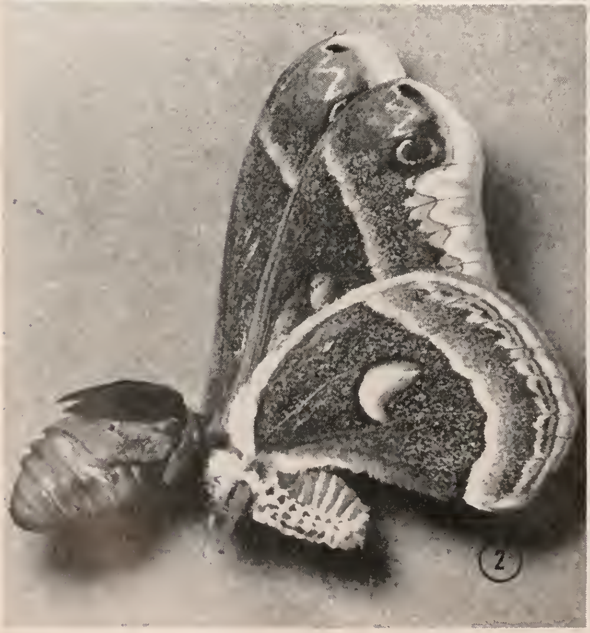
The experiments now under consideration differed from the preceding in that the pupal partners possessed endocrinologically active brains and were therefore able to initiate adult development within a few days after being placed at room temperature.

Forty preparations were assembled, of which ten soon died and were discarded. Table II summarizes the several types of experiments that were performed. In each of the 30 viable preparations the pupal partner initiated adult development within ten days at 25° C. Attention is directed to the effects of the parabiosis on the course of this development.

When the headless partner was a male or female Polyphemus moth, the pupa







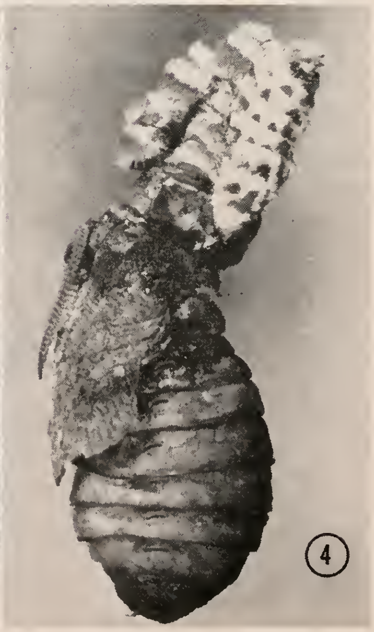


FIGURE 1. A headless male Cecropia moth is here joined in parabiosis with a previously chilled pupa of the Polyphemus silkworm.

FIGURE 2. After three weeks at 25° C. the pupa has molted to form a second pupal stage showing only traces of adult characters. The old pupal cuticle has been removed.

FIGURE 3. The abdomen of a male Cecropia moth is joined in parabiosis with a previously chilled Cecropia pupa.

FIGURE 4. After three weeks at 25° C. the pupa has molted to form a second pupal stage showing only traces of adult characters. The old pupal cuticle has been removed. The adult abdomen has molted its adult cuticle which is here shown partially peeled away.

underwent normal adult development. The same was true in 7 of 8 preparations in which the headless partner was a female Cecropia moth. But in all ten preparations in which the headless partner was a male Cecropia moth, the pupa metamorphosed, not into a normal moth, but into a creature which retained large areas of pupal cuticle (Fig. 2). It seems necessary to conclude that a male Cecropia moth, though headless and without any corpora allata, can somehow favor the release of juvenile hormone within the parabiotic preparation.

In the analysis of these experiments we have centered attention on the effects of the parabiosis on the pupal partner. But, what about the developmental response of the other half of the combination—the headless moth? For present purposes suffice it to say that in about a third of the preparations the developmental reaction of the pupa spread to the adult partner and caused the latter to molt. The molt extended over both the thorax and abdomen, but never included the wings. The old adult cuticle was detached from the underlying epidermis and replaced by a smooth new cuticle which was of adult type, except for the generalized absence of scales or hairs.

3. *Parabiosis between previously chilled pupae and moth thoraces and abdomens*

Each of a series of ten male Cecropia moths was beheaded and subdivided into thorax and abdomen. The two parts were then joined in parabiosis with previously chilled Polyphemus pupae and placed at 25° C.

Within the following month all the surviving pupae which had been joined to adult thoraces emerged as normal moths. By contrast, all of the surviving pupae which had been joined to adult abdomens developed into moths retaining prominent pupal characteristics. This result demonstrated that juvenile hormone activity was associated with the abdomen of male Cecropia moths and that the thorax was inert in this respect.

4. *Juvenile hormone activity in relation to the species and sex of the moth abdomen*

Table III summarizes a large series of experiments which were performed to test the abdomens of male and female saturniid moths belonging to five different species. A total of 110 parabiotic preparations was established, of which 83 were viable.

Here again we see that the abdomens of male Cecropia moths always provoked strong positive tests for juvenile hormone. The same result was obtained for the abdomens of male Cynthia moths. The only other species to give a positive test was one of six abdomens of male Orizaba moths. The abdomens of both male and female Polyphemus moths were inactive; the same was true for the closely related Pernyi moth and for most males and all females of Orizaba.

Juvenile hormone activity in these adult insects is therefore a species-specific characteristic which is seen most prominently in Cecropia and Cynthia moths. Even here, it is a sex-linked characteristic being routinely encountered in males and only rarely, if at all, in female moths.

Though the outcome is strikingly dependent on the species and sex of the adult component in the parabiotic preparations, the result is seemingly independent of the species or sex of the pupal partner. This fact is emphasized in Table III where we see that male Cecropia abdomens gave uniformly positive tests when

TABLE III

Parabiosis between previously chilled pupal and moth abdomens

Adult abdomen	Species of pupal partner	Number of preparations	Effects on pupal partner
♀ Polyphemus	♂ or ♀ Polyphemus	4	Formed normal moths
♂ Polyphemus	♂ or ♀ Polyphemus	6	Formed normal moths
♀ Pernyi	♂ or ♀ Polyphemus	2	Formed normal moths
♂ Pernyi	♂ or ♀ Polyphemus	4	Formed normal moths
♀ Orizaba	♂ or ♀ Polyphemus	3	Formed normal moths
♂ Orizaba	♂ or ♀ Polyphemus	6	5 formed normal moths 1 formed moth retaining a patch of pupal cuticle on thoracic tergum
♀ Cecropia	♂ or ♀ Polyphemus	3	Formed normal moths
	♀ Cecropia	5	Formed normal moths
♂ Cecropia	♂ or ♀ Polyphemus	21	Developed into moths retaining many pupal characters
	♀ Cecropia	13	Developed into moths retaining many pupal characters
	♂ Cecropia	4	Developed into moths retaining many pupal characters
♀ Cynthia	♂ or ♀ Polyphemus	6	Formed normal adults
♂ Cynthia	♂ or ♀ Polyphemus	6	Developed into moths retaining many pupal characters

joined to pupae of male Cecropia, female Cecropia, male Polyphemus, or female Polyphemus.

In about a third of the preparations the adult abdomen underwent a molt in synchrony with the development of the pupal partner (Figs. 4 and 7). This phenomenon will be considered in further detail in the Discussion.

5. *Temporary parabiosis between previously chilled pupae and abdomens of male Cecropia moths*

How long must a pupa be joined to a male Cecropia abdomen before it will give a positive test for juvenile hormone? This question was studied in six preparations in which the initial parabiosis was disassembled after a certain time and the adult abdomen grafted to a fresh pupa.

The results were as follows: When the initial parabiosis was for only one day, the pupa developed into a normal moth, whereas the second pupal partner developed into a mixture of pupa and adult. But when the first parabiosis was extended to five days, then both the first and the second pupal partners gave positive tests for juvenile hormone. A transient blood connection with the adult is therefore sufficient.

In additional experiments testing this point it appears that the parabiosis must continue until the pupal partner actually initiates its adult development; in the above-mentioned experiments this occurred on the fourth day. If the pupa requires more than four days to initiate its development, then the parabiosis must be continued until it does so.

6. *Effects of allatectomy*

The results show that abdomens of male Cecropia and Cynthia moths are somehow able to cause a positive test for juvenile hormone when joined to pupal partners. Since the adult corpora allata are cephalic structures, the moth abdomen did not appear to be a reasonable candidate for the secretion of juvenile hormone. Attention therefore centered on the corpora allata in the pupal partner. Despite the fact that pupal corpora allata are known to be inactive (Williams, 1961), it seemed possible that they might be "turned on" by some influence arising in the moth abdomen. In order to test this possibility, the series of experiments, summarized in Table IV, was carried out.

Corpora allata were excised from four previously chilled Cecropia pupae. Then, each allatectomized pupa was joined to a headless male Cecropia moth. Though the preparations now contained no corpora allata, a positive test for juvenile hormone was obtained in each case. This result leaves no reasonable doubt that the source of the juvenile hormone was the adult abdomen itself. This conclusion was tested as follows:

The corpora allata and corpora cardiaca were excised from six chilled male Cecropia pupae which were then placed at 25° C. and allowed to develop into adult moths. Three of these allatectomized moths were beheaded and joined to previously chilled pupae; in the other three, the moth abdomens were excised and used in the parabiosis. As recorded in Table IV, all six preparations now gave negative tests for juvenile hormone. This shows that the accumulation of juvenile hormone by the abdomen is dependent on its synthesis and secretion by the corpora allata in the head.

In a further test, the corpora allata and corpora cardiaca were excised from two previously chilled male Cecropia pupae and two pairs of "loose" pupal corpora

Table IV

Parabiosis between previously chilled pupae and male Cecropia moths; either the pupal or adult partner had been allatectomized

Adult component	Pupal partner	Number of experiments	Effects on pupal partner
Headless ♂ Cecropia	Allatectomized ♀ Cecropia	4	Developed into moths retaining many pupal characters
Headless ♂ Cecropia (allatectomized in pupal stage)	♂ Cecropia	3	Formed normal moths
♂ Cecropia abdomen (allatectomized in pupal stage)	♂ Cecropia	3	Formed normal moths
♂ Cecropia abdomen (allatectomized in pupal stage and two pairs of pupal corpora allata re-implanted into head)	♂ Cecropia	2	Developed into moths retaining many pupal characters

allata-cardiaca were re-implanted into the head. When the moths emerged, their abdomens were joined to chilled pupae. The latter gave positive pupal assays for juvenile hormone (Table IV). This indicates that implanted corpora allata can substitute for the animal's own corpora allata in the secretion of juvenile hormone.

7. *Elution of juvenile hormone from abdominal tissues of male Cecropia moths*

On the basis of the experiments just considered, it seems necessary to conclude that the juvenile hormone is secreted by the corpora allata in the head of the adult moth and that, in the case of male Cecropia and Cynthia moths, the abdomen is somehow able to bind and accumulate substantial amounts of hormone. We are led to the prediction that the abdomen of the male Cecropia moth must contain a depot of juvenile hormone. A direct test of this inference was made as follows:

The abdomens of several male Cecropia moths were dissected in Ringer's solution. Various tissues and organs were removed, rinsed, and tested by implantation under a plastic window at the tip of the abdomen of previously chilled pupae. The results, summarized in Table V, show that positive assays for juvenile hormone were obtained in certain cases after the implantation of adult fat-body, abdominal nerve cords, or vas deferens. The positive test in each case was a minimal reaction in that pupal cuticle was re-formed only under the abdominal window at the site of injury (Williams, 1961). A number of other adult organs, including a solution of yolk obtained from unfertilized Cecropia eggs, gave negative tests.

TABLE V

*Tests for juvenile hormone in abdominal tissues and organs of adult male Cecropia moths**

Adult tissue	Number of preparations	Results of tests
Fragment of fat-body	3	2 positive; 1 negative
Abdominal ganglia and connectives	4	1 positive; 3 negative
Vas deferens	5	1 positive; 4 negative
Testes	2	Negative
Gut	1	Negative
Solution of egg yolk from adult female	3	Negative

* The tissues or organs were implanted into the tip of the abdomen of previously chilled Cecropia or Polyphemus pupae. The test was scored as positive when the pupa formed a moth retaining pupal characters.

DISCUSSION

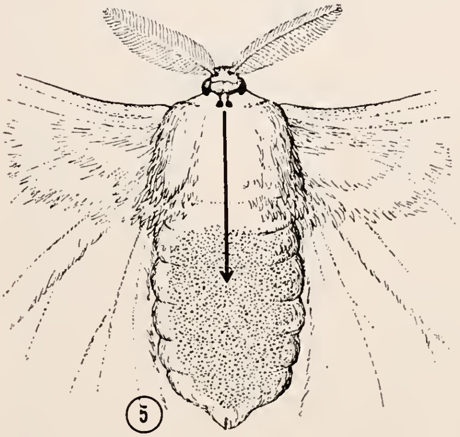
1. *The sequestering of juvenile hormone*

The experimental results reveal the surprising fact that the abdomens of male Cecropia and Cynthia moths contain a cache of juvenile hormone. The hormone is found in the most prominent tissue of the abdomen—the fat-body—and also in at least two other tissues, the nerve cord and the vas deferens. Moreover, the hormone can be eluted from these tissues when the latter are either implanted or joined to a test pupa by way of the hemolymph. This shows that the sequestered hormone is in some sort of dynamic equilibrium with the circulating blood.

Though the hormone accumulates in the abdomen, it is synthesized by the corpora allata in the head (Fig. 5). So, if the corpora allata are excised prior to the final week of adult development, then, as diagrammed in Figure 6, no hormone accumulates in the abdomen.

It will be recalled that the corpora allata are inactive in the pupa and that this inactivity persists during two-thirds of adult development (Williams, 1961). Then, on or about the fourteenth day of adult development, the corpora allata recover the activity they had lost months earlier at the time of pupation. Yet, strange to say, the juvenile hormone has no known function in the adults of these short-lived Lepidoptera. Allatectomized Cecropia pupae develop into normal male and female moths which, when cross-mated, give rise to fertile eggs (Williams, 1959).

Despite persistent uncertainties as to the role of juvenile hormone in these adult insects, we can confidently say that the hormone is sequestered in the abdominal tissues of male Cecropia and Cynthia moths. This process presumably begins after the corpora allata regain their activity during the final week of adult development, and continues during the entire week of adult life (Williams, 1961). The abdomen, in effect, serves as an extraction chamber which, over a period of about two weeks, binds and accumulates the hormone which is continuously secreted by the corpora allata.



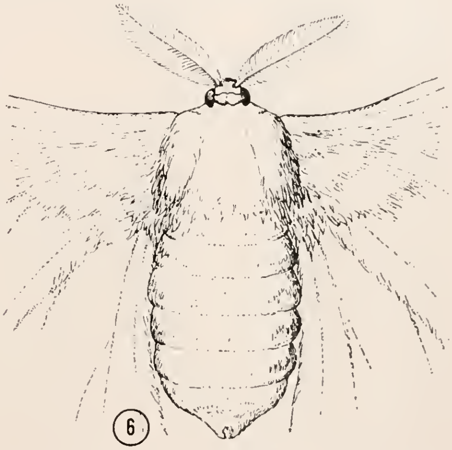


FIGURE 5. As indicated by the stippled area, the juvenile hormone which accumulates in the abdomen of the male Cecropia moth is synthesized and secreted by the corpora allata in the head.

FIGURE 6. If the corpora allata are removed from the head, then no hormone accumulates in the abdomen. (Figures 5 and 6 are used with the permission of the *Scientific American.*)

2. *The species- and sex-specific accumulation of juvenile hormone*

The accumulation of hormone takes place in male Cecropia and Cynthia moths, but only rarely in females. Yet the corpora allata of female moths are just as active in secreting juvenile hormone as are those of males (Williams, 1959). Moreover, the accumulation of juvenile hormone occurs in neither male nor female

Pernyi or Polyphemus moths despite the fact that the adult corpora allata of these insects are extremely active in the secretion of juvenile hormone (Williams, 1959).

For these several reasons it is amply evident that those moths which fail to accumulate the hormone must dispose of it in some manner, presumably by converting it into inactive products. Whatever this inactivating mechanism may be, it is obviously curtailed in the case of male Cecropia and Cynthia moths.

3. *The induced molting of adult moths*

Among the pterygote insects only the Ephemeroptera are known to molt as adults—a phenomenon which has recently been subjected to detailed study by Taylor and Richards (1963). Yet, as demonstrated in the present study, one can often cause an adult moth to molt by joining it in parabiosis with a pupal partner.

The molt consisted of a detachment and retraction of the epidermis from the adult cuticle and the secretion of a new cuticle; in no case did the moth make any effort to escape from the exuviae. The molting of the adult began in synchrony with that of the pupal partner. When the latter was a diapausing pupa and failed to initiate development (Table I), then no trace of molting occurred in the adult. This shows that the molting of the moth was a response to the ecdyson secreted by the pupal prothoracic glands and conveyed to the moth in the pupal blood.

Manifestly, the cells and tissues of the moth retain the potential for further development and molting when supplied with ecdyson. The failure of adult insects to molt is therefore directly attributable to the breakdown of their prothoracic glands and the consequent lack of ecdyson.

In the parabiotic preparations molting occurred in only about one-third of the moths. This capricious result was apparently due to uncontrollable variations in the circulation of blood between the pupal and adult partners. The blood connection which one can establish at the anterior end of a headless moth is, perforce, of small diameter. And when an adult abdomen was used, the blood flow from the pupa was commonly impaired by the herniation into the pupa of the moth ovaries and the fluid-filled rectal sac. In many of the adult partners these circumstances apparently precluded the build-up of a threshold titer of the ecdyson.

In most preparations the molting of the moth was incomplete and one could not remove the loose cuticle because of its persistent attachments to the spiracles and genitalia. However, in occasional preparations the molt was complete and the old cuticle could be easily peeled from the abdomen.

A preparation of this type is shown in Figure 7. The entire adult cuticle has been molted, including the intricate cuticle of the male genitalia. The new cuticle preserves the smooth texture and pale tan pigmentation of normal adult cuticle, the only striking difference being the virtual absence of scales or hairs. The naked character of the new adult cuticle was especially prominent in Cecropia and was apparently due to the depletion of "scale mother cells" which during the formation of the first adult cuticle were transformed into lifeless scales and sockets (Henke, 1946). However, in the case of Polyphemus or Pernyi abdomens, the new adult cuticle commonly showed substantial clusters of scales along the dorsum of the abdominal segments; this apparently signifies the presence in these species of a reserve supply of scale and socket "stem cells."

In all of the preparations that molted, a new adult cuticle formed, irrespective of whether the molt occurred in the presence or absence of juvenile hormone. The new cuticle showed no trace of the reappearance of pupal characteristics even when the molt took place in the presence of sufficient juvenile hormone to cause the pupal partner to form a second pupal stage. This result is therefore different from that reported for *Rhodnius* where, according to Wigglesworth (1940, 1958), a partial "reversal of metamorphosis" occurs when an adult is caused to molt in the presence of juvenile hormone.

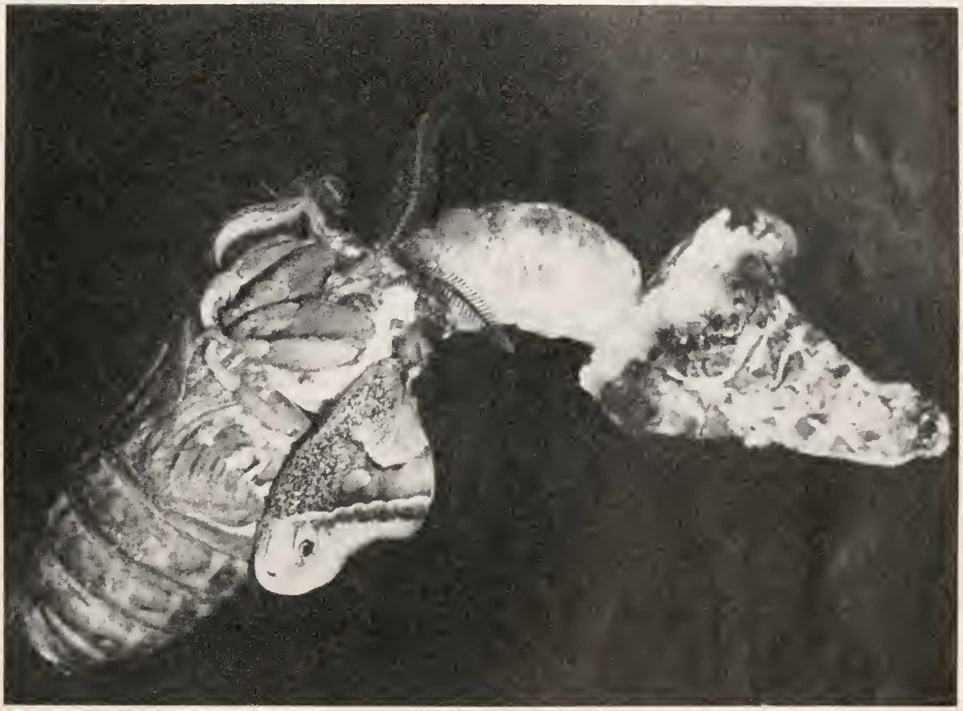


FIGURE 7. The abdomen of a male Cecropia moth was joined with a previously chilled pupa of the Cecropia silkworm. Here, after four weeks at 25° C., the pupa has transformed into a moth, preserving numerous pupal characters. Meanwhile, the moth abdomen has fully molted its adult cuticle and formed a new cuticle of adult type, except for the absence of scales and hairs.

4. *Prolongation of adult life*

In all the species used in the present study the moths are unable to feed because of the absence of functional mouth parts. Therefore, they rarely survive at room temperature for more than a week. And yet, when beheaded and joined to diapausing pupae, these same moths routinely survived for many weeks, during which time one could elicit leg movements and the flapping of the wings. Even the most moribund moth, within a few hours of death, reacted to parabiosis with renewed vitality and longevity.

Manifestly, the moths are sustained by the fluids and substrates which are continuously transfused *via* the pupal blood. This shows that the moths normally die of desiccation and nutritional deficiencies, and that the adult cells and tissues are capable of a far longer life-span than they can ordinarily display.

5. *Sexual reflexes of headless moths and isolated abdomens*

Many species of insects show exaggerated sexual reflexes following decapitation—an effect attributable to a release from the normal inhibitory influences of the cephalic ganglia (Roeder *et al.*, 1960). Effects of this sort were particularly prominent in parabiotic preparations where the decapitated individual survived for up to ten weeks.

The effects were most spectacular in the headless males or isolated male abdomens of Pernyi and Cecropia. Here, immediately after recovery from anesthesia, the individual initiated lively motion of both its abdomen and claspers, precisely as in normal mating. This behavior usually continued for several days, and was accompanied by the discharge of one or more spermatophores from the extended aedeagus. This curious behavior on the part of the headless male moth or male abdomen was far less conspicuous in Orizaba and totally lacking in Polyphemus.

In general, the effects of decapitation were not as prominent in the case of female moths. One generally observed a great increase in the tone of the intersegmental muscles and a downward flexion of the tip of the abdomen as in normal egg-laying. However, no eggs were actually oviposited, even though the abdomen was full of them.

6. *The mimicking of brain hormone by juvenile hormone*

According to experiments reported previously (Williams, 1959), the implantation of active corpora allata can cause the initiation of development in a certain proportion of brainless diapausing pupae. On further analysis, the conclusion was reached that under certain conditions the juvenile hormone can activate the prothoracic glands and in this sense mimic the function of the brain hormone.

These earlier results are strikingly reminiscent of those recorded in Table I. Here, 4 of 9 diapausing Cecropia pupae were caused to initiate development by joining them in parabiosis with headless male Cecropia moths. The net effect in this case, as in the experiments reported four years ago, was the termination of diapause and the production of pupal-adult monstrosities.

The stimulation of development in the pupal partner, as noted in Table I, was observed when diapausing pupae were joined to male Cecropia moths which, as we have seen, contain a rich depot of juvenile hormone. In the absence of sequestered hormone, headless female Cecropia moths, as well as headless Polyphemus and Pernyi of both sexes, gave negative tests in all cases.

Consequently, the experiments summarized in Table I are additional evidence that the juvenile hormone can mimic the brain hormone in its ability to turn on the prothoracic glands.

SUMMARY

1. Pupae joined in parabiosis with headless male Cecropia moths behave as if they have received an injection of juvenile hormone. They develop, not into

normal moths, but into creatures which show a mixture of pupal and adult characters.

2. By diverse experiments it was possible to show that the juvenile hormone comes from the moth abdomen and that the abdominal tissues of male Cecropia moths contain a rich depot of juvenile hormone.

3. In the moth, itself, the hormone is synthesized by the corpora allata in the head and is progressively bound and sequestered by the abdominal tissues. If the corpora allata are removed from the head, then no hormone accumulates in the abdomen.

4. The accumulation of juvenile hormone in the abdominal tissues occurs in male Cecropia and Cynthia moths, but not in females. In the case of certain related species of saturniid moths (Polyphemus, Pernyi and Orizaba), neither sex is ordinarily able to accumulate the hormone, despite the fact that they have very active corpora allata.

5. The failure to accumulate the hormone points to some unknown means for its inactivation; these agencies are evidently curtailed or by-passed in the case of male Cecropia and Cynthia moths which accumulate large amounts of hormone.

6. Adult moths are frequently caused to molt when joined to non-diapausing pupae and thereby supplied with ecdyson. A new adult cuticle forms which is deficient in scales and hairs. Adults molting in the presence of high concentrations of juvenile hormone show no reappearance of pupal characters or any sign of a "reversal of metamorphosis."

7. When the adult tissues are continuously perfused with pupal blood, the life-span of the moths is greatly prolonged. This shows that, in the absence of functional mouthparts, the moth normally dies of desiccation and starvation rather than from the intrinsic biological death of the tissues themselves.

8. In experiments involving the parabiosis of diapausing pupae with moths containing a depot of juvenile hormone, additional evidence was obtained that juvenile hormone can turn on the prothoracic glands and, in this sense, mimic the brain hormone.

LITERATURE CITED

HENKE, K., 1946. Ueber die verschiedenen Zellteilungsvorgänge in der Entwicklung des beschuppten Flügelepithels der Mehlmotte *Ephestia kühniella*. *Biol. Zbl.*, **65**: 120–135.

ROEDER, K. D., L. TOZIAN AND E. A. WEIANT, 1960. Endogenous nerve activity and behavior in the mantis and cockroach. *J. Insect Physiol.*, **4**: 45–62.

TAYLOR, R. L., AND A. G. RICHARDS, 1963. The subimaginal cuticle of a mayfly (*Callibaetis* sp.). *Ann. Entomol. Soc. Am.* (in press).

WIGGLESWORTH, V. B., 1940. The determination of characters at metamorphosis in *Rhodnius prolixus* (Hemiptera). *J. Exp. Biol.*, **17**: 201–222.

WIGGLESWORTH, V. B., 1958. Some methods for assaying extracts of the juvenile hormone in insects. *J. Insect. Physiol.*, **2**: 73–84.

WILLIAMS, C. M., 1952a. Morphogenesis and the metamorphosis of insects. *The Harvey Lectures*, **47**: 126–155.

WILLIAMS, C. M., 1952b. Physiology of insect diapause. IV. The brain and prothoracic glands as an endocrine system in the Cecropia silkworm. *Biol. Bull.*, **103**: 120–138.

WILLIAMS, C. M., 1959. The juvenile hormone. I. Endocrine activity of the corpora allata of the adult Cecropia silkworm. *Biol. Bull.*, **116**: 323–338.

WILLIAMS, C. M., 1961. The juvenile hormone. II. Its role in the endocrine control of molting, pupation, and adult development in the Cecropia silkworm. *Biol. Bull.*, **121**: 572–585.

FORMATION OF ENDODERM FROM ECTODERM IN CORDYLOPHORA

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There has been no satisfactory resolution for the problem of the extent to which one type of differential animal cell may be converted to that of another type. In one guise or another this question has come into prominence a number of times during the past half century. Several decades ago the issue was whether "dedifferentiation" followed by "redifferentiation" in a new direction is possible. The observations of H. V. Wilson (1907, 1911a, 1911b) on reconstitution from dissociated sponges and hydroids stimulated one phase of interest in the problem. Contributions to the discussion were made by many outstanding biologists of that time (*e.g.*, J. S. Huxley, C. M. Child, J. Loeb, T. H. Morgan, etc.). Later, experiments with cultured tissues led to the general observation that rapidly proliferating tissues lose their characteristic morphological appearance. This stimulated more research and thinking on the "dedifferentiation-redifferentiation" issue. The major aspects of the current attitudes in this field have been reviewed by Trinkaus (1956) and Grobstein (1959).

In general there are two schools of thought: one, which maintains that cell types are irreversibly fixed and that one type of cell cannot be transformed to another (except within a rather narrow "modulatory" range) and the other, which claims that at least some types of cell transformation of a more radical sort (metaplasia) are possible. A number of well substantiated cases of metaplasia have been described in recent years. Our knowledge of Wolffian regeneration (formation of a lens from the iris of the eye) has been expanded by L. S. Stone and collaborators (1952). Fell and Mellanby (1953) and Weiss and James (1955) have demonstrated that the ordinarily squamous epithelium from the skin of 6-day chick embryos may be converted to a mucous secreting columnar epithelium by exposure to excess vitamin A. The tissues of the chorio-allantoic membrane of chick embryos may change in response to a variety of conditions (Moscona, 1959, 1960; Moscona and Carneckas, 1959). Other cases may be cited. There also are well substantiated cases which indicate that some tissues and some cell types are quite stable and do not transform to other types. This is especially true of so-called terminal tissues, *i.e.*, tissues which have achieved an extreme state of functional maturity (possibly involving eventual cell sloughing) or extreme cell specialization. Ordinarily such cells do not divide. There remains a broad area in which the picture is not clear and it seems as though the best approach to the problem is to state it in some form as "which cell types in which tissues are stable and which are sufficiently unstable so that they may undergo metaplasia under desig-

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nated circumstances?" and then to design experiments which may answer this question.

A complicating factor for this general problem is the possible existence, in many organisms and tissues, of reserve cells with "embryonic" capabilities. In some situations, as in the case of regeneration of amphibian limbs, the importance of reserve cells has been discounted (Butler, 1935). In other cases (*i.e.*, interstitial cells (I-cells) of hydroids, amebocytes of sponges and neoblasts of flat worms) the reserve cell notion still has wide acceptance (Brien and Reniers-Decoen, 1955; Tardent, 1960; Burnett, 1962). Because of this situation, answers to the questions raised above may be equivocal unless the experiments are designed to deal with progenies of single cells of known origin (*i.e.*, clones). Unfortunately, the technical aspects of cloning cells have not yet been perfected for most cells coming from a wide range of tissues and organisms. In fact, the application of cloning procedures is still quite restricted. Since this is the case our present approaches must be less direct and less decisive. This communication deals with an experiment of the latter sort. While not unequivocal, it makes a contribution to our understanding of cell capacities in hydroids.

Three papers, dealing with reconstitution of hydroids, may be cited as typical of the attitudes one encounters in such studies. Gilchrist (1937) found that ectoderm and endoderm could be separated quite readily in scyphistomae of *Aurelia*. The mesoglea is sufficiently thick in these animals so that it may be cut with a knife, leaving ectoderm with one half and endoderm with the other. The endodermal sheets formed small ciliated balls which lived for days but did not reconstitute an individual. The ectoderm, on the other hand, did form an individual complete with an endodermal layer. Gilchrist felt that the new inner layer must have been formed by the interstitial cells.[2]

Beadle and Booth (1938) were, *inter alia*, concerned with the question of whether I-cells, largely restricted to ectoderm in *Cordylophora*, can form endodermal elements. To this end they separated ectoderm from endoderm in reconstitution masses made from the coenosarc of *Cordylophora*. Four masses of supposedly pure ectoderm were isolated. One of these developed a small hydranth and sections of two of the others revealed that they had begun to form an inner layer. The authors were uncertain of the nature of the inner layer in these two cases, since the cells were not typically endoderm. However, they felt that the fully reconstituted hydranth came from a case in which (p. 312) ". . . a small amount of endoderm must have been included. . . ." These are the two typical "explanations": I-cells formed the new tissue or some cells from the tissue in question "must have been included"! To complete the cycle I may cite Papenfuss and Bokenham (1939) who were unable to obtain reconstitution with either isolated ectoderm or endoderm from *Hydra*. These authors suggest the possibility that Gilchrist did not remove all of the endoderm from his supposed ectodermal isolates!

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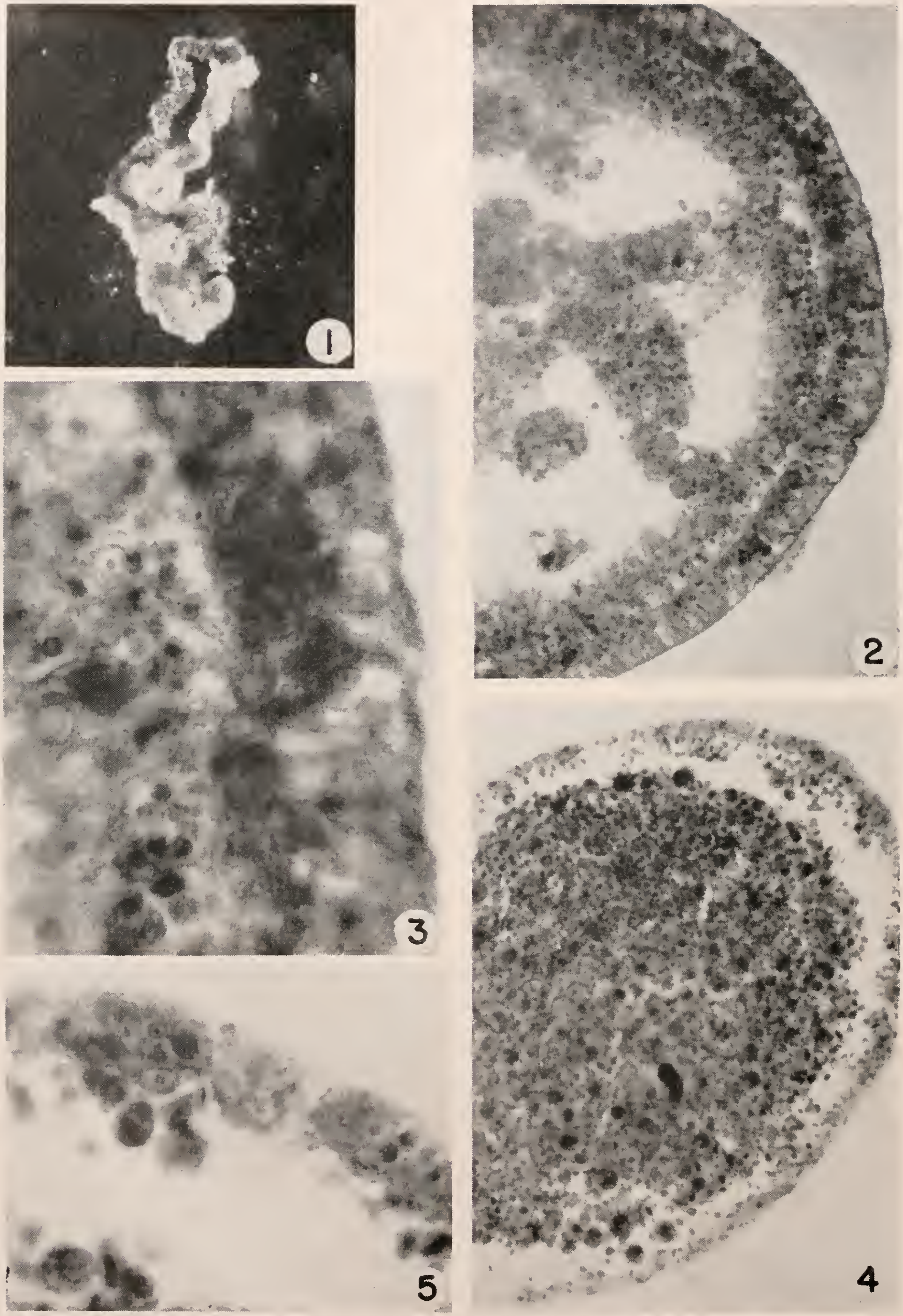
[2] Gilchrist did not include a histological study of his material with his account. Steinberg (1963) repeated Gilchrist's experiments with *Aurelia* scyphystomae and, from her histological study, found that these animals do not have typical interstitial cells. She did confirm the finding that a new endodermal layer formed from isolated ectoderm and presented evidence that proliferating ectodermal cells give rise to amoebocytic cells which then form a typical endodermal layer. The amoebocytes themselves do not divide actively; instead, the somatic ectodermal cells show considerable mitotic activity during the time that amoebocytes increase in number.



FIGURES 1–5.

Beadle and Booth dealt with only four masses of supposedly endoderm-free ectoderm, and an inner layer of some sort was formed in three of these. The original question which concerned Beadle and Booth was whether I-cells of the ectoderm can form endoderm. Their conclusion that an endodermal layer can form only when the ectoderm is contaminated with former endoderm cells and that I-cells in the ectodermal layer cannot form endodermal elements seems to be somewhat shaky in view of their scanty evidence. Before a judgment can be made on the chief problem about the developmental capacity of the ectodermal cells it is important to establish more firmly the answer to the first question: does an endodermal layer form only from pre-existing endodermal cells or, put the other way, can an endodermal layer form from uncontaminated ectoderm? This question has been subjected to close scrutiny with the *Cordylophora lacustris* found near Woods Hole as the test object. The results do not support Beadle and Booth but, instead, indicate that an individual, complete wth an endodermal layer, may be reconstituted from isolated ectoderm of *Cordylophora*.

MATERIAL AND METHODS

The *Cordylophora* used for these experiments was collected during July and August of two successive summers at the edge of a fresh-water pond. The best spot for collection was near a wooden run-off at one end of the pond. A small clump of the mature colony was placed in a jar with some of the pond water, taken to the laboratory and kept cool and aerated in the original pond water. Only fresh material (*i.e.,* in the laboratory no more than three days) was used in the experiments.

While the *Cordylophora* lived in fresh water, it was found that a more favorable medium for reconstitution of masses of coenosarc was 25–30% sea water (Beadle and Booth, 1938, used 50% sea water). The sea water was filtered, diluted with either tap or distilled water and then pasteurized. The pasteurized medium was cooled and aerated before use.

Healthy, relatively straight stems were selected. The distal hydranth was cut away, and the coenosarc was pushed out of the perisarc by means of a curved bit of thin glass rod or a bit of shaped Tygon tubing which was held in a pair of forceps. Usually the gleaming white ectoderm separated from the pigmented endoderm (yellow or orange) very readily (Fig. 1). An exposure of the stem to the 25% sea water for 5–30 minutes before the extrusion greatly facilitated the separation of layers.

FIGURE 1. Newly extruded coenosarc of *Cordylophora*. Note the separation of ectoderm and endoderm (darker tissue) in the upper portion of the preparation.

FIGURE 2. Section through 10-hour mass of equal parts of ectoderm and endoderm. Note the complete separation of the two layers and the presence of a thin mesogleal layer between the two. Note also that cells are intact.

FIGURE 3. High power view of same section as in Figure 2. Note the inclusions in the endothermal cells (at left).

FIGURE 4. Section through 10-hour mass of pure ectoderm. Note the thin outer wall and highly fragmented and disorganized contents of the hollowed sphere. Compare with Figure 2.

FIGURE 5. High power view of same section as in Figure 4. Note the character of the cells of the wall; also the interstitial cell adhering to its inner surface.

Beadle and Booth found that neutral red was taken up selectively by the endoderm and took advantage of this to distinguish the two layers. Since the natural pigmentation of the endoderm in our animals was so distinctive and the layers separated quite readily, no supplementary treatment was deemed necessary. Separation was completed with the aid of cataract knives and the ectoderm was removed to a clean dish for further inspection. The tissue was cut into small pieces (0.1–0.2 mm. in diameter) and each piece was carefully inspected, at high magnifications of the dissecting microscope (50–90 ×), for contaminating endoderm. Any pigmented material was removed from the ectoderm. Reflected and transmitted as well as oblique lighting were used to insure the identification and elimination of the orange endodermal cells. These could be seen most favorably against a white background. In this way there was reasonable certainty that all endodermal cells were removed. Several of the small masses were then heaped together and allowed to fuse and form a single mass. Masses were transferred to fresh medium in small Stender dishes and kept on a shaded part of the laboratory table.

Despite all of the precautions taken there was always the possibility that one or two endodermal cells could escape our scrutiny and be included inadvertently. In order to assess the consequences of this possibility we set up control masses of ectoderm to which minute traces (no more than 3–5 cells) of endoderm were deliberately added. In addition other controls with varying amounts of endoderm (up to 50%) were used.

The table-top temperature, which was recorded for each experiment, varied from 19° to 22° C. Ectodermal masses were fixed in Bouin's fixative at various times following isolation; these were embedded in paraffin and sectioned at 5–10 μ. Toluidine blue at pH 5 proved to be a favorable stain.

RESULTS

A total of 117 viable masses of *Cordylophora* coensarc was prepared for this study. Forty of these consisted of pure ectoderm (*i.e.,* no endoderm discernible with high magnifications of the dissecting microscope), 40 had deliberately included traces of endoderm, and in the rest endoderm comprised up to 50% of the total tissue mass. Thirty-five masses were fixed at various times after isolation.

Pieces of extruded coenosarc fused readily and, in most cases, formed a smooth sphere. In favorable cases the sphere became hollow within five to 10 hours after isolation. Spheres which did not become hollow did not reconstitute, but remained in this inactive condition for days. The center gradually became opaque but the surface layer still retained the refractile appearance usually associated with living tissue. The hollowing-out process was noted by Beadle and Booth, who conjectured about the activity of ectoderm in transporting fluid to the interior of the mass.

Almost 50% of the "pure" ectoderm masses reconstituted an inner layer (as judged by the presence of clearly discernible flagellar motion in the enteron) and most of these (9) formed a complete hydranth in 5–9 days. The rest (4) remained as stolonic growths which persisted without change for a long time. Essentially the same behavior was noted in masses which had a minute trace of endoderm. In both of these classes of isolates many (circa 50%) of the masses remained inactive (see above), and did not reconstitute. In sharp contrast all but one of the 50:50 masses reconstituted a hydranth (one formed a stolonic growth); none were inactive.

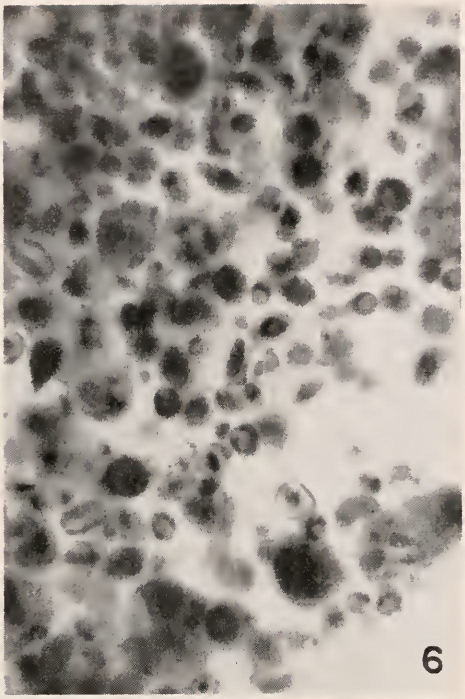
Most of the larger 50:50 masses formed a hydranth very quickly, within 30–36 hours. The average time for hydranth formation was 2–3 days. This is the average for all size classes; it was noted, however, that larger masses reconstituted a hydranth more quickly than smaller ones. When the masses are distributed into two groups, those larger and those smaller than 0.2 mm., the average time for hydranth formation for the group of larger isolates (6 masses) was 1.1 days and that for the smaller (13 masses) was 2.4 days. Moreover, the hydranths formed by most of the smaller mass were incomplete and had fewer tentacles than normal. A number of the large masses, on the other hand, developed more than one hydranth. (See Chalkley, 1945, for relation between initial mass and morphogenesis in *Hydra*.)

Histological Observations

Our histological study was made on 25 masses (10 were lost or were not satisfactory for study) which were fixed at various times during reconstitution. Twelve of these were masses of supposedly pure ectoderm, eight were masses which had a trace of endoderm, and five consisted of equal amounts of ectoderm and endoderm.

One of the first questions which required an answer was whether an occasional endoderm cell could be identified in the midst of a mass of ectoderm. Examination of sections of whole stems revealed that most of the endoderm cells were full of large inclusions of various sorts. This was the situation except in the hydranth and in the region immediately proximal to it where inclusion-free endoderm cells may be found. However, these regions were cut away before coenosarc was expressed and, therefore, most of the endoderm with which we are concerned was characterized by the presence of many inclusions. Ectoderm cells are not free of inclusion but rarely have more than one or two per cell. On the basis of this criterion we could ascertain that supposedly endoderm-free masses had no cells with more than 1–2 inclusions while a few cells with numerous inclusions could be identified in masses deliberately contaminated with traces of endoderm. The combination of this observation with the ease of identification of even single orange cells (endoderm) in the living mass when high magnifications of the dissection microscope were used provides reasonable confidence that our masses were endoderm-free. I doubt that one can make a stronger statement in a situation of this sort. Neither criterion is absolute; masses which are allowed to live can be, at the best, only good approximations of those sectioned.

The histological picture derived from masses of equal amounts of ectoderm and endoderm is quite clear and simple. The two layers rapidly sort out and, by the end of ten hours, form a definite inner endodermal layer which is separated from the ectoderm by a distinct basement-membrane-like mesoglea (Figs. 2 and 3). Cells of both layers are intact and retain the appearance which is typical of an intact stem. When the hydranth is elaborated many of the endoderm cells acquire the characteristics of typical secreting cells of the digestive portion of a hydranth. The two salient features are: cells remain intact, and sorting into two distinct layers is rapid.



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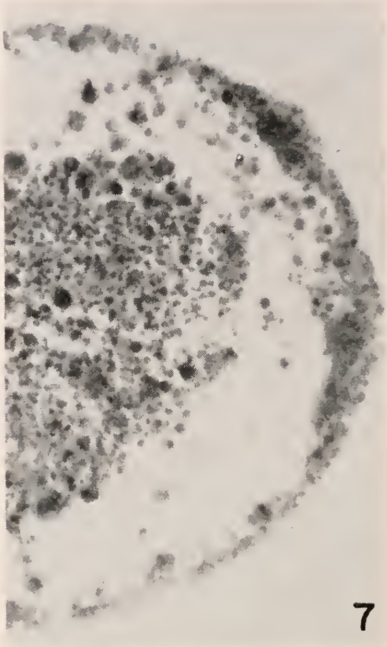

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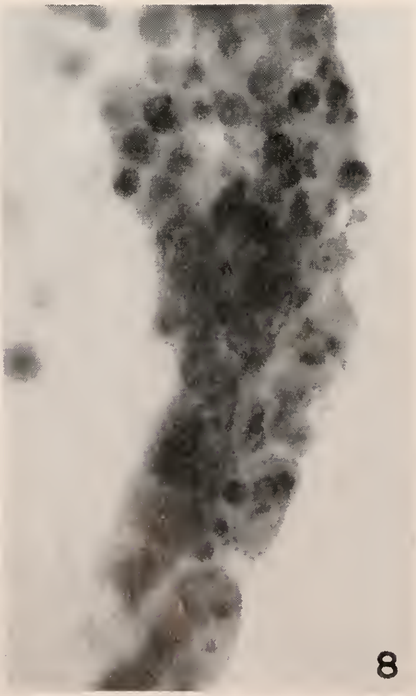

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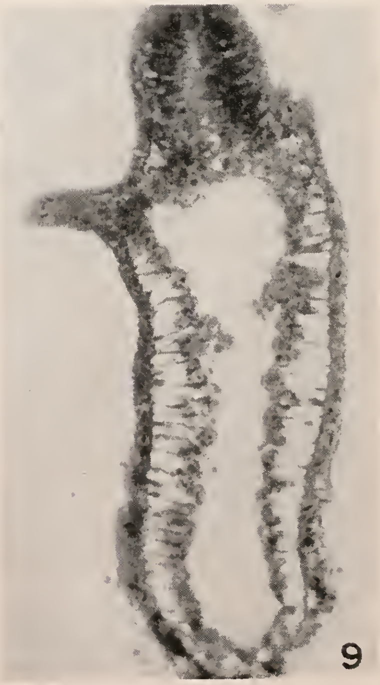

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Figures 6–9.

Since there was no observable difference between masses of "pure" ectoderm and those with traces of endoderm these two groups shall be treated together. Masses in these categories are characterized by a dramatic phenomenon. Sections of isolates which were fixed 1–2 hours after preparation revealed a picture which was quite similar to that seen in the ectoderm of sectioned intact stems. Somatic ectoderm cells and a number of I-cells could be distinguished clearly. By 8–10 hours all of the active masses were hollow and sections revealed that the relatively thin outer wall consisted of a single layer of small ectodermal cells. In isolates which were fixed at 10 hours or somewhat later, the contents of the sphere included, for the most part, cell fragments and scattered I-cells and relatively few recognizable intact somatic cells (Figs. 4, 5, 6). In some specimens it could be seen that a few I-cells were closely adherent to the inner surface of the cells of the sphere's wall (Fig. 5).

At this stage, it was evident that many small basophilic cells were scattered through the cell debris. Some of the somatic cell nuclei were pycnotic (this was confirmed, in another series, with iron hematoxylin-stained material) but there were not enough degenerating nuclei to account for the total number of initial somatic cells. On the other hand there was a marked increase in the number of small basophilic cells (I-cells) but no indication of mitotic activity.

During subsequent days the picture was essentially the same as for the 10-hour masses except that more I-cells accumulated on the inner surface of the wall (Figs. 7 and 8). By the fourth day a complete inner layer of enlarged cells was found in all masses which had shown signs of internal flagellar beating prior to fixation. Mitotic figures were not evident during the interval from 10 hours to 4 days. By the time a hydranth had formed (5–9 days), the inner layer was typically endodermal (Fig. 9). During the later phases of this process (4 days and following) the endodermal cells in many of the masses contained inclusions. The decrease in amount of cell debris in the hollow of the mass leads to the suspicion that the cells were phagocytizing the debris.

It is difficult to draw definite conclusions from static evidence of the sort presented above. There is the possibility that the picture reconstructed from this particular series is not typical. There is no certainty that the new endodermal layer forms from the interstitial cells which are seen adhering to the inner surface of the wall of the spheres. Surviving somatic ectodermal cells could, conceivably, make this inner layer. Efforts are under way to obtain more rigorous evidence which may shed light on these problems. However, one aspect of the original question seems reasonably clear: an endodermal layer does form from pure ectoderm. This point has been established more firmly by the following additional observations.

Figure 6. High power view (oil) of fragmented cellular material in interior of 24-hour ectodermal isolate. Note at least one small basophilic cell, many enucleated cell fragments and inclusions, and occasional (upper left) intact somatic cell. This photograph is from the same section as Figure 7.

Figure 7. Section of 24-hour ectodermal isolate. Note two groups of intensely basophilic cells adhering to the inner surface of the sphere's wall.

Figure 8. High power view of one of two groups of basophilic cells shown in Figure 7. Note the typical interstitial cell characteristics of these cells.

Figure 9. Section through a fully formed hydranth which has formed from an ectodermal isolate.

Two-Step Operation

Several *facts* could be derived from the observations described above. Within the first ten hours after separation of a mass of ectoderm a hollow sphere was formed, the wall of the sphere consisted of small ectodermal cells, all contaminating inadvertent or deliberately included endoderm cells were in the center of the sphere and were fragmented (or fragmenting) along with the rest of the ectoderm. In addition to this there were no I-cells *in* the ectodermal wall, but a few may have been adhering to its inner surface. An operation was carried out which could take advantage of the condition at 10 hours. Eleven ectodermal masses were prepared in the usual way. After 10–12 hours these were opened up and the inner contents were extruded and discarded. The inner surface of the walls was scraped clean of adherent material. The walls were then cut up and allowed to fuse together to form 5 second-generation reconstitution masses.

All of the second-generation masses became hollow and, by the sixth day after the second step of the operation, all five of them had an endodermal layer which could be detected by the definite flagellar movements which were readily discernible in the intact masses. Three of these elongated and formed stolonic growths while the other two formed typical tentacled hydranths by the end of the seventh day. This last experiment removes any doubt that an endodermal layer can form from isolated ectoderm of *Cordylophora*.

Discussion

The observations presented above indicate that the answer to the question of whether an endodermal layer can form from ectoderm of *Cordylophora* is different than that arrived at by Beadle and Booth. On the basis of a careful scrutiny of the material I feel confident that endoderm-free preparations of *Cordylophora* can reconstitute an endodermal layer. In addition to the histological confirmation of the absence of endoderm there is the two-stage operation which, in my mind, provides the most rigorous evidence in support of this contention.

Equally rigorous evidence about the ectodermal cell type which forms the endoderm is not at hand. Present observations do not allow us to ascertain whether only pre-existing interstitial cells or former somatic ectodermal cells contribute to the new layer. We hope to present additional evidence on this point in a subsequent publication. However, there is evidence from other forms which indicates that similar metaplastic changes may take place in the absence of I-cells. Normandin (1960) has reported (without details) that ectoderm-free endoderm of *Hydra* will reconstitute a new ectoderm when the endodermal isolate is free of I-cells. In my laboratory S. N. Steinberg (1963) has reinvestigated Gilchrist's observations with ectoderm from scyphistomae of *Aurelia*. She has confirmed the formation of an endodermal layer in the ectodermal isolates but her histological studies have failed to reveal an ectodermal cell type which has the characteristics of an I-cell. The nature of I-cells and their possible contribution to morphogenetic processes should, at the present, be regarded with a great many reservations.

The loss of tissue integrity and the accompanying cellular fragmentation which are typical of the ectodermal isolates of *Cordylophora* are striking phenomena. They appear to be very similar to the "histolysis" of muscle cells which has been noted in regenerating urodele limbs (Butler, 1935; Thornton, 1938; Hay, 1959) where the muscle nuclei with some of the cell cytoplasm pinch off and acquire the characteristics of regeneration cells while the enucleated parts of the cell degenerate and are eventually removed by phagocytes. Cellular fragmentation of *Cordylophora* ectoderm seems to be related to an imbalance in the relative amounts of ectoderm and endoderm. In several cases in which single small masses (0.1 mm. in diameter) of endoderm were included with considerably larger amounts of ectoderm, tissue integrity was maintained locally, and a mesoglea was quickly re-established at the site of contact between endoderm and ectoderm. Ectoderm adjacent to this region of normal tissue association underwent the usual fragmentation. It is interesting to speculate that the key to the fragmentation may reside in whether or not a mesoglea persists or is re-formed. Isolated ectoderm of *Aurelia* scyphystomae does not undergo fragmentation; the thick mesoglea retains its usual relation to the cells. There seems to be a relationship between the persistence of a mesoglea or its rapid re-establishment and retention of cell integrity. This may merely be a coincidence, but it is an intriguing one.

SUMMARY

Within ten hours after isolation of ectodermal masses of *Cordylophora lacustris,* a hollow sphere is formed in which the outer wall consists of a single layer of relatively small cells and most of the inner cells are fragmented and disorganized. During the course of 5–9 days an entire hydroid forms—complete with an endodermal layer. A two-stage operation, in which only the thin outer walls of 10-hour isolates were kept for further observation, also resulted in the formation of new endodermal layers. While it is not clear whether interstitial or somatic ectoderm cells are involved, it is evident that some ectodermal derivative forms the new layer. The possible relationship between cell fragmentation and absence of a mesoglea is noted.

LITERATURE CITED

Beadle, L. C., and F. A. Booth, 1938. The reorganization of tissue masses of *Cordylophora lacustris* and the effect of oral cone grafts, with supplementary observations on *Obelia gelatinosa*. *J. Exp. Biol.*, **15**: 303–326.

Brien, P., and M. Reniers-Decoen, 1955. La signification des cellules interstitielles des hydres d'eau douce et le problème de la réserve embryonnaire. *Bull. Biol. France et Belg.*, **89**: 258–325.

Burnett, A. L., 1962. The maintenance of form in *Hydra*. *In:* Regeneration. Twentieth Growth Symposium. Ronald Press, New York. Pp. 27–52.

Butler, E. G., 1935. Studies on limb regeneration in X-rayed *Amblystoma* larvae. *Anat. Rec.*, **62**: 295–307.

Chalkley, H. W., 1945. Quantitative relation between the number of organized centers and tissue volume in regenerating masses of minced body sections of *Hydra*. *J. Nat. Cancer Inst.*, **6**: 191–195.

Fell, H. B., and E. Mellanby, 1953. Metaplasia produced in culture of chick ectoderm by high vitamin A. *J. Physiol.*, **119**: 470–488.

Gilchrist, F. G., 1937. Budding and locomotion in the scyphistomas of *Aurelia*. *Biol. Bull.*, **72**: 99–124.

Grobstein, C., 1959. Differentiation of Vertebrate Cells. *In:* The Cell. Brachet and Mirsky, Editors. Academic Press, New York. Pp. 437–496.

Hay, E. D., 1959. Electron microscopic observations of muscle differentiation in regenerating *Amblystoma* limbs. *Devel. Biol.*, **1**: 555–585.

Moscona, A., 1959. Squamous metaplasia and keratinization of chorionic epithelium of the chick embryo in egg and in culture. *Devel. Biol.*, **1**: 1–23.

Moscona, A., 1960. Metaplastic changes in the chorioallantoic membrane. *Transpl. Bull.,* **26**: 120–124.

Moscona, A., and Z. I. Carneckas, 1959. Etiology of keratogenic metaplasia in the chorioallantoic membrane. *Science,* **129**: 1743–1744.

Normandin, D. K., 1960. Regeneration of *Hydra* from the endoderm. *Science,* **132**: 678.

Papenfuss, E. J., and N. A. H. Bokenham, 1939. The fate of the ectoderm and endoderm of *Hydra* when cultured independently. *Biol. Bull.,* **76**: 1–6.

Steinberg, Sonia N., 1963. The regeneration of whole polyps from ectodermal fragments of scyphistoma larvae of *Aurelia aurita. Biol. Bull.,* **124**: 337–343.

Stone, L. S., 1952. An experimental study of the inhibition and release of lens regeneration in adult eyes of *Triturus v. viridescens. J. Exp. Zool.,* **121**: 181–224.

Tardent, P., 1960. Principles governing the process of regeneration in hydroids. *In:* Developing Cell Systems and their Controls. Eighteenth Growth Symposium. Ronald Press, New York. Pp. 21–43.

Thornton, C. R., 1938. The histogenesis of muscle in the regenerating forelimb of larval *Amblystoma punctatum. J. Morph.,* **62**: 17–47.

Trinkaus, J. P., 1956. The differentiation of tissue cells. *Amer. Nat.,* **90**: 273–288.

Weiss, P., and R. James, 1955. Skin metaplasia *in vitro* induced by brief exposure to vitamin A. *Exp. Cell Res., Suppl.* **3**: 381–394.

Wilson, H. V., 1907. On some phenomena of coalescence and regeneration in sponges. *J. Exp. Zool.,* **5**: 245–258.

Wilson, H. V., 1911a. On the behavior of dissociated cells in Hydroids, *Alcyonaria,* and *Asterias. J. Exp. Zool.,* **11**: 281–338.

Wilson, H. V., 1911b. Development of sponges from dissociated tissue cells. *Bull. (for 1910) U. S. Bur. Fisheries (Doc. 750),* **30**: 1–30.

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